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Spermidine in the Bacterial Cell

By U. BACHRACH AND I. COHEN

Department of Clinical Microbiology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

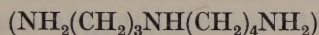
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SUMMARY

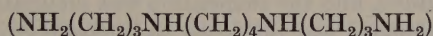
The concentration of spermidine in extracts of various micro-organisms was determined; *Neurospora crassa* contained the highest concentrations of spermidine, while in *Pseudomonas aeruginosa*, *Escherichia coli* and *Saccharomyces cerevisiae* the concentration of spermidine was lower. Growing organisms and cell-free extracts of the micro-organisms tested were able to synthesize [^{14}C]spermidine from DL-[2- ^{14}C]methionine. Cell-free extracts of *Pseudomonas aeruginosa* were most active in this respect. [^{14}C]Spermidine formed by growing *Bacillus subtilis* was localized in both the protoplasts and the bacterial cell walls. The kinetics of the adsorption of [^{14}C]spermidine on to cell walls and protoplasts of *B. subtilis* were also studied. The interpretation of these observations and their relation to the antibacterial effect and growth-promoting activity of spermidine are discussed.

INTRODUCTION

The polyamines spermidine



and spermine



are widely distributed in nature. They have been found in animal tissues (Dudley & Rosenheim, 1925; Dudley, Rosenheim & Starling, 1927; Harrison, 1931; Rosenthal & Tabor, 1956), in micro-organisms, especially Gram-negative bacteria (Herbst, Weaver & Keister, 1958; Weaver & Herbst, 1958), and in some viruses (Ames, Dubin & Rosenthal, 1958; Bawden & Pirie, 1959; Ames & Dubin, 1960). Conjugated polyamines such as acetylated spermidine were found in *Escherichia coli* (Dubin & Rosenthal, 1960*a*); a spermidine-glutathione conjugate from *E. coli* has also been described (Dubin & Rosenthal, 1960*b*). Other conjugates of polyamines have been found in a phospholipid from human malignant tumours (Kosaki, Ikoda, Kotani, Nakagawa & Saka, 1958) and in spider poisons (Fischer & Bohn, 1957*a*).

Very little is known about the biosynthesis of polyamines. The condensation of putrescine with methionine in the presence of adenosine triphosphate and magnesium sulphate has been proposed as a pathway for the biosynthesis of spermidine in *Escherichia coli* (Tabor, Rosenthal & Tabor, 1957), and in *Neurospora crassa* (Greene, 1957). The enzymes involved in this condensation reaction have been purified from *E. coli* (Tabor, Rosenthal & Tabor, 1958; Tabor & Tabor, 1960). Weaver & Herbst (1958) have found that *Haemophilus parainfluenzae* produced spermidine from

propane-1:3-diamine. Quantitative determinations of spermidine in animal tissues were made by Rosenthal & Tabor (1956) and Fischer & Bohn (1957*b*). Very little, however, is known about the quantities of polyamines found in micro-organisms.

The purpose of the present investigation was to determine quantitatively the amount of spermidine present in extracts of various micro-organisms, to isolate spermidine from different constituents of the bacterial cell, and to study the mechanism of spermidine absorption to the bacterial cell.

METHODS

Organisms. Strains of the following micro-organisms were used: *Bacillus subtilis* (ATCC 6633); *Pseudomonas aeruginosa* (Bachrach, 1957); *Escherichia coli* 59; *Neurospora crassa* 34455; *Saccharomyces cerevisiae* 81. All these strains were from the collection of the Department of Clinical Microbiology, Jerusalem.

Media. *Neurospora crassa* was grown in the defined medium of Fries described by Nason & Evans (1953). All the other micro-organisms were grown in the medium of Davis (1949) after adjustment to pH 7.0.

Conditions of growth. The micro-organisms were grown in quantities of 1 l. liquid medium in a 3 l. flask agitated on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). After 20 hr. of incubation at 37° the organisms were harvested by centrifugation (Sharples supercentrifuge, or MSE high-speed centrifuge) and washed three times with physiological saline.

Preparation of cell-free extracts. The micro-organisms were subjected to the action of a 10 kcyc. Raytheon sonic oscillator for 25 min. Debris and intact organisms were removed by high-speed centrifugation (13,000 g for 10 min.).

Preparation of protoplasts. These were prepared according to Fitz-James (1958). Organisms were washed three times with saline and suspended in 0.1 M-phosphate buffer (pH 6.1) containing 0.5 M-sucrose and 0.016 M-magnesium sulphate. Lysozyme (1 mg./ml.) was then added, and the suspension was incubated at 37° for 30–60 min. The formation of protoplasts was observed by phase-contrast microscopy. Whole organisms were removed by centrifugation (3000 g) and the protoplasts separated from the supernatant fluid by a second centrifugation (10,000 g).

Preparation of cell walls. These were prepared according to Salton & Horne (1951). Organisms were subjected to the action of a Mickle tissue disintegrator for 60 min. Unbroken organisms were removed by centrifugation (3000 g) and the cell walls separated by a second centrifugation (10,000 g). The purity of the preparation was checked by electron microscopy.

Chemicals. Spermidine phosphate was obtained from Hoffman-La-Roche (Basle, Switzerland). Putrescine dihydrochloride, DL-methionine and adenosine triphosphate (ATP), were the products of Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). 1-Fluoro-2:4-dinitrobenzene was purchased from L. Light and Co. Ltd (Colnbrook, Bucks, England). DL-[2-¹⁴C]methionine was obtained from California Corporation (Los Angeles, U.S.A.).

Spermidine estimations. Paper chromatography was the most convenient method for the qualitative and quantitative estimation of the amines. Butanol + acetic acid + water (50 + 25 + 25 by vol.; Baker, Harborne & Ollis, 1952) was the solvent system used. The papers were sprayed with 0.2% (w/v) ninhydrin in butanol.

Quantitative determinations were carried out by eluting the coloured complex (Giri, Radhakrishnan & Vaidyanathan, 1952).

Ion exchange chromatography. A solution of 10% (w/v) trichloroacetic acid solution was added to an equal volume of the sample to be examined. The precipitate formed was removed by centrifugation and the residual trichloroacetic acid extracted with ether. Samples of the supernatant fluid were then passed through a column of Dowex 50 H⁺ (6 mm. diameter; 60 mm. high) and the spermidine eluted from this column by a gradient of HCl (prepared by the dropwise addition of 2.5 N-HCl to 300 ml. water in a mixing vessel) at a flow rate of 1 ml./min. Fractions of 3.5 ml. were collected in an automatic fraction collector (LKB-Produkter, Stockholm, Sweden) and the eluate containing spermidine (fraction between 210–270 ml.) was concentrated in vacuum, neutralized and estimated colorimetrically as the 2:4-dinitrophenyl derivative (Rosenthal & Tabor, 1956).

Steam distillation. Samples containing spermidine were steam distilled in a Markham still after the addition of potassium hydroxide (Dudley, Rosenheim & Rosenheim, 1924).

Protein estimations. Proteins were estimated by the spectrophotometric method of Layne (1957) with a Unicam Model SP 500 Spectrophotometer (Unicam Instruments Ltd, Cambridge).

Radioactivity estimations. Spermidine was identified by paper chromatography and the radioactive areas on the paper were counted either directly with a mica end-window Geiger-Müller tube, or eluted with water and subsequently assayed for radioactivity by means of an end-window lead-shielded Geiger-Müller tube (Tracerlab). Spermidine was also separated by the ion exchange method, converted into the 2:4-dinitrophenyl derivative, extracted with ethyl acetate and assayed for radioactivity by means of a Geiger-Müller counter.

RESULTS

Quantitative determinations of polyamines in various micro-organisms

Extracts of micro-organisms obtained after disruption by sonic oscillation were examined for polyamines by paper chromatography. The polyamines detected were separated by the ion-exchange method and assayed colorimetrically. As shown in Fig. 1, 21 µg. spermidine were found per mg. protein in an extract of *Neurospora crassa*. The concentration of spermidine in extracts of *Pseudomonas aeruginosa* was appreciably lower (12.5 µg./mg. protein). The lowest concentration was obtained with *Saccharomyces cerevisiae* (only 7 µg./mg. protein).

Synthesis of [¹⁴C] spermidine by growing organisms and by cell-free extracts

The micro-organisms were grown for 20 hr. at 37°, harvested, washed with saline and resuspended in 4.0 ml. of medium to which phosphate buffer and [2-¹⁴C]methionine had been added. After further incubation, the organisms were harvested and disrupted. The spermidine in the extracts was separated by ion-exchange chromatography, and its radioactivity determined. *Neurospora crassa* contained the highest concentration of [¹⁴C]spermidine, while the lowest concentration was found in *Pseudomonas aeruginosa* (Fig. 2).

After ascertaining that growing organisms were capable of producing spermidine

from putrescine, it was attempted to synthesize this polyamine by extracts obtained from the various micro-organisms. Putrescine, [2- 14 C]methionine, ATP and magnesium sulphate were incubated with the various extracts. The spermidine formed was separated by ion-exchange chromatography and its radioactivity determined. The highest concentrations of [14 C]spermidine were obtained with extracts of *Pseudomonas aeruginosa* (Fig. 3).

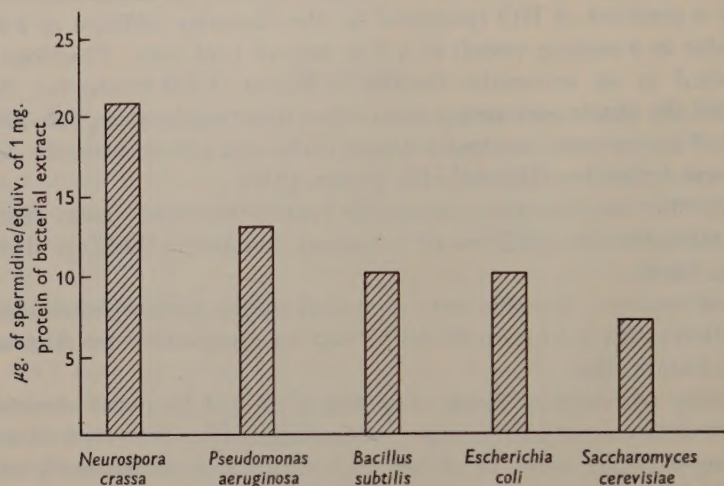


Fig. 1. Spermidine in extracts of various micro-organisms.

Localization of spermidine in *Bacillus subtilis*

Bacillus subtilis was chosen for study because of the detailed techniques elaborated for the separation of its cellular constituents. The localization of spermidine in the cell walls and protoplasts was examined by incubating intact bacilli in a medium fortified with either [2- 14 C]methionine or [14 C]spermidine prepared from [2- 14 C]methionine in the presence of *Pseudomonas aeruginosa* as described above. *B. subtilis* was grown in 2000 ml. medium at 37° for 20 hr. The bacteria were harvested, washed and

Table 1. [14 C]Spermidine in cell walls and protoplasts of *Bacillus subtilis* grown in the presence of DL-[2- 14 C]methionine or [14 C]spermidine

Bacteria were grown for 20 hr. at 37°, harvested and resuspended in 10.0 ml. of medium supplemented with 2.5 µmole DL-[2- 14 C]methionine (1.5×10^5 c.p.m.) or 1.0 ml. of [14 C]spermidine (5×10^5 c.p.m.) and incubated for another 14 hr. The [14 C]spermidine in the cell walls and protoplasts of these bacteria was determined.

Preparation	mg. of protein/ml. of preparation	mg. of dry wt./ml. of preparation	c.p.m./ml. of preparation	c.p.m./mg.	
				Protein	Dry wt.
Cell walls incubated with:					
[¹⁴ C]Methionine	0.9	10.5	2.160	2.400	206
[¹⁴ C]Spermidine	1.7	19.8	2.082	1.225	105
Protoplasts incubated with:					
[¹⁴ C]Methionine	2.9	5.8	243	84	42
[¹⁴ C]Spermidine	3.1	6.2	220	71	36

resuspended in 10 ml. of medium to which $[2-^{14}\text{C}]$ methionine had been added. After another 14 hr. of incubation the bacteria were again harvested and protoplasts and cell walls prepared. The $[^{14}\text{C}]$ spermidine in the samples was separated by steam distillation. The distillate was concentrated and examined by paper chromatography.

The amount of $[^{14}\text{C}]$ spermidine bound to an equivalent of 1 mg. dry wt. of the cell wall was higher than that bound to the same equivalent of protoplast. The amount of $[^{14}\text{C}]$ spermidine localized in the cell walls of *Bacillus subtilis* was also higher when $[^{14}\text{C}]$ methionine was added to the growing organisms (Table 1).

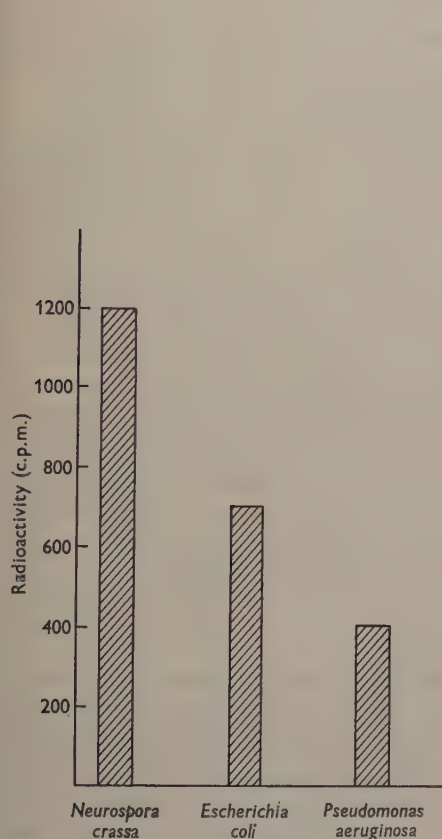


Fig. 2

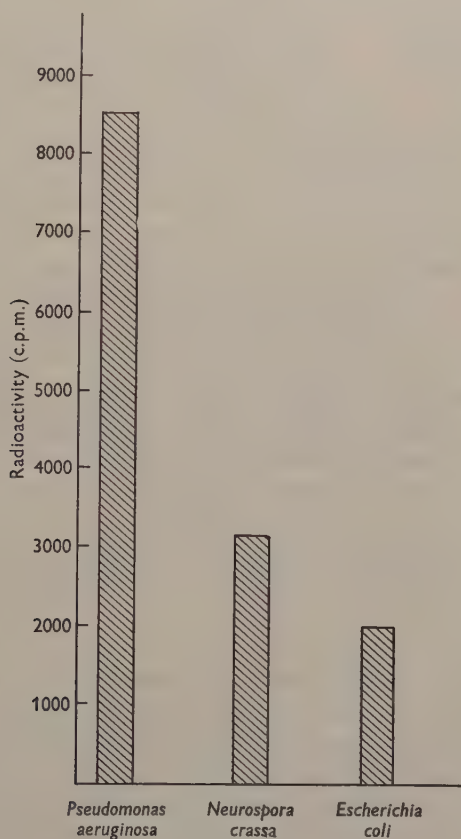


Fig. 3

Fig. 2. Synthesis of $[^{14}\text{C}]$ spermidine by growing cells of various micro-organisms. The bacteria were grown for 20 hr. at 37° , harvested and resuspended in 4.0 ml. of medium, supplemented with 3.0 ml. of 0.067 M-phosphate buffer pH 7.9; $2.5 \mu\text{mole}$ of DL- $[2-^{14}\text{C}]$ -methionine (1.5×10^6 c.p.m.) and incubated for another 14 hr. The cells were then harvested, disrupted and the concentration of $[^{14}\text{C}]$ spermidine in the extracts determined.

Fig. 3. Synthesis of $[^{14}\text{C}]$ spermidine by cell-free extracts of various micro-organisms. The reaction mixtures contained: 1.0 ml. of 0.067 M-phosphate buffer pH 8.0; $1.0 \mu\text{mole}$ of DL- $[2-^{14}\text{C}]$ methionine (0.6×10^6 c.p.m.); $1.0 \mu\text{mole}$ of putrescine; $30 \mu\text{mole}$ of MgSO_4 ; $2.5 \mu\text{mole}$ of ATP and 3.0 ml. of cell-free extract (equiv. of 50 mg. of protein). Incubation was carried out at 37° for 60 min.

Binding of [^{14}C]spermidine by the cell walls and protoplasts of Bacillus subtilis

Having established that [^{14}C]spermidine was bound to the cell wall of *Bacillus subtilis* and to a smaller degree to its protoplast, the kinetics of this reaction were studied. Figure 4 shows that cell walls of *B. subtilis* rapidly bound [^{14}C]spermidine (prepared biologically by *Pseudomonas aeruginosa*) as indicated by the decrease in radioactivity of the supernatant fluid, and the increase of radioactivity in the precipitate (i.e. cell walls). The binding of [^{14}C]spermidine by protoplasts is shown in Fig. 5.

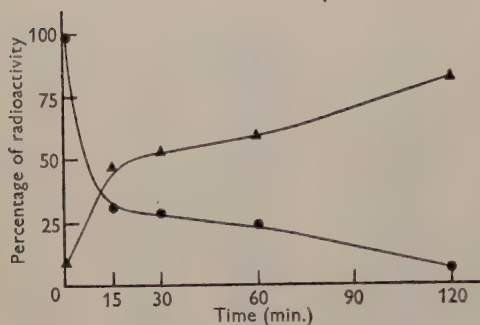


Fig. 4

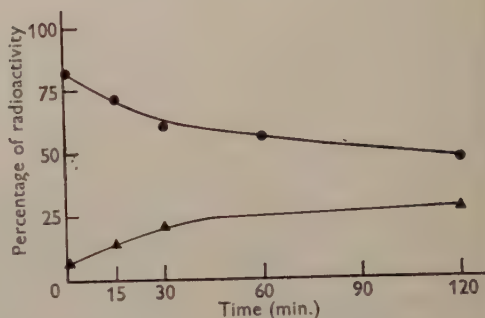


Fig. 5

Fig. 4. Binding of [^{14}C]spermidine by cell walls of *Bacillus subtilis*. The reaction mixture contained 1.0 ml. of [^{14}C]spermidine (5.0×10^3 c.p.m.) and 4.0 ml. of cell wall suspension (equiv. of 5.2 mg. of protein or 61 mg. dry wt.). Samples were taken at the time intervals indicated, centrifuged and the radioactivity of the supernatants, ●, and the precipitates, ▲, determined.

Fig. 5. Binding of [^{14}C]spermidine by protoplasts of *Bacillus subtilis*. Experimental conditions were as given in Fig. 4, but 4.0 ml. of protoplast suspension (equiv. of 10.0 mg. of protein or 20 mg. dry wt.) was employed. ●, Supernatant; ▲, precipitate.

DISCUSSION

The amine content of *Escherichia coli* depends on growth conditions such as the composition and pH value of the growth medium. In the absence of spermidine in the growth medium, 1 g. wet wt. organism was found to contain about 200 μg . spermidine (Dubin & Rosenthal, 1960a). Brewers yeasts contain 130 μg . spermidine/g. wet wt. (Fischer & Bohn, 1957b). These amounts are similar to those found in rat or rabbit livers (Rosenthal & Tabor, 1956). The use of wet weights of organisms for the comparison of the amounts of spermidine in different organisms, however, might lead to considerable errors. We therefore used the protein content of the examined bacterial extracts as a criterion in the evaluation of our results. The amount of spermidine in the extract is not equal to the total spermidine content of an organism, for insoluble constituents of the cells may retain a considerable amount of this compound which cannot be completely extracted even with 0.1N-hydrochloric acid (Razin & Rozansky, 1959). The results represented in Fig. 1 indicate that spermidine is present in similar concentrations in the extracts of the Gram-positive and Gram-negative organisms tested. Extracts of *Pseudomonas aeruginosa* produced more [^{14}C]spermidine from [2- ^{14}C]methionine than the equivalent extracts of *Neurospora crassa* and *E. coli* (Fig. 2). The quantities of [^{14}C]spermidine which accumulated

within the organisms grown on [2-¹⁴C]methionine were smaller than those produced by extracts (Fig. 3). This is not surprising and might be explained by the limited capacity of the whole organism to store the polyamine. Decomposition of spermidine by *P. aeruginosa* (Razin, Gery & Bachrach, 1959) may also cause a decrease in its intracellular concentration.

The attachment of polyamines to bacteria was described by Razin & Rozansky (1959), and Mager (1959) studied the binding of spermidine to protoplasts. The receptors on the cell wall might be located in the plastic film of lipoprotein described by Mitchell (1959) or in some bacteria in the lipoprotein particles like those responsible for the adsorption of bacteriophages (Anderson, 1960). The active sites in the protoplasts might be associated with its ribonucleic acid content (Cohen & Lichtenstein, 1960) or with the cytoplasmic lipoprotein layer (Luria, 1960).

Experiments described in this paper indicate that *Bacillus subtilis* grown in the presence of methionine or spermidine contained spermidine in cell walls and protoplasts. Cohen & Lichtenstein (1960) found that polyamines were attached to ribosome particles of *Escherichia coli* prior to cell disruption, and were not bound during or after the formation of the extract. If this concept is general, one could exclude the possibility of redistribution of the polyamines during the disintegration, and assume that the location of the polyamines in disintegrated and intact cells is the same.

Spermine and spermidine are known to inhibit the growth of various bacteria (Gurevitch, Rozansky, Weber, Brzezinsky & Eckerling, 1951; Rozansky, Bachrach & Grossowicz, 1954) and fungi (Razin, Cohen, Rozansky, 1958). As both sensitive and resistant bacteria absorb these polyamines (Razin & Rozansky, 1959), the selective toxicity is not explained by absorption alone. Detoxification of spermidine by acetylation (Dubin & Rosenthal, 1960*a*) does not seem to solve the problem, for acetylated polyamines were also found in *Staphylococcus aureus* (Rosenthal & Dubin, 1960), which is sensitive to spermidine. Recent studies on the mode of action of streptomycin indicate that its initial uptake by the bacterial cell occurs outside the cytoplasmic membrane. The secondary uptake is caused by the cytoplasmic membrane, resulting in changes of the cell permeability (Anand & Davis, 1960). Furthermore, streptomycin-resistant bacteria do not bind streptomycin on their cytoplasmic membrane (Anand, Davis & Armitage, 1960). Streptomycin resembles spermidine in its basic property; both are bound to acidic components like nucleic acids (Cohen, 1946; Fraser & Mahler, 1958; Felsenfeld & Huang, 1960) and their biological activity is counteracted by constituents of serum. One may speculate that the polyamines inhibit the growth of sensitive micro-organisms by interfering with the normal function, synthesis, or permeability barrier, of the cytoplasmic membrane.

The growth-promoting effect of polyamines for certain bacteria (Herbst & Glinos, 1955; Martin, Pelczar & Hansen, 1952; Sneath, 1955; Traub, Mager & Grossowicz, 1955; Kihara & Snell, 1957) remains still to be explained. The degradation of spermidine to β -alanine and other compounds by *Pseudomonas aeruginosa* (Razin, Gery & Bachrach, 1958, 1959; Bachrach, 1960) and by *Mycobacterium smegmatis* (Bachrach, Persky & Razin, 1960) appears to apply to these two micro-organisms and does not explain the growth-promoting activity for other bacteria. It seems possible that polyamines may enhance bacterial growth by affecting the permeability barrier of the cells (e.g. by preventing leakage of nutrients from the cell). Similar effects have been obtained with mitochondria (Tabor, 1960; Herbst & Whitherspoon, 1960) and bacterial protoplasts (Mager, 1959).

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A New Genus of the Actinomycetales: *Micropolyspora* gen. nov.

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SUMMARY

Three filamentous micro-organisms are described. They are typical Actinomycetales forming branching hyphae, 1.5 μ or less in diameter, which are differentiated into a substrate (primary) mycelium and an aerial (secondary) mycelium. They have an unusual mode of sporulation since they form chains of conidia both on the substrate and on the aerial mycelium. A new genus, *Micropolyspora*, is proposed. The type species is *M. brevicatena*. The new genus is part of the family Actinomycetaceae. The abolition of the family Streptomycetaceae is proposed.

INTRODUCTION

Actinomycetes are filamentous bacteria which are often considered as intermediate between the true bacteria and the fungi. According to the latest edition of *Bergey's Manual* (1957) these organisms are grouped under the order Actinomycetales, which is subdivided into four families as follows:

- | | |
|---|-------------------|
| I. Mycelium rudimentary or absent; no spores formed | Mycobacteriaceae |
| II. True mycelium produced | |
| A. Spores formed, but not in sporangia | |
| 1. Spores formed by fragmentation of mycelium | Actinomycetaceae |
| 2. Vegetative mycelium normally remains undivided | Streptomycetaceae |
| B. Spores formed in sporangia | Actinoplanaceae |

Bergey's Manual recognizes three genera in the family Streptomycetaceae.

They are characterized as follows:

- | | |
|---|--------------------------|
| I. Conidia produced in aerial hyphae in chains | <i>Streptomyces</i> |
| II. Conidia produced terminally and singly on short sporophores | |
| A. No growth between 50° and 65° | <i>Micromonospora</i> |
| B. Growth occurs between 50° and 65° | <i>Thermoactinomyces</i> |

Since the publication of the 1957 edition of *Bergey's Manual*, other genera have been described, to include organisms which have novel modes of spore formation. These include: (1) *Waksmania* Lechevalier & Lechevalier (1957), in which pairs of spores are formed longitudinally on the aerial mycelium. The same genus, under the name *Microbispora*, was described at the same time in Japan by Nonomura &

Ohara (1957). (2) *Thermopolyspora*, in which short chains of spores are formed on the aerial mycelium of thermophilic organisms (Henssen, 1957).

The present paper reports the isolation and description of a type of actinomycete which produces spore-bearing substrate mycelia and aerial mycelia. The name proposed for the new genus is *Micropolyspora*. The type species is *M. brevicatena*. Two strains have been isolated and are deposited in the culture collection of the Institute of Microbiology, Rutgers, The State University, New Brunswick, N.J. (nos. 1084 and 1086). In addition another strain of the same genus was also isolated (no. 1085). This strain has cultural properties which differentiate it from the other two. Until more strains of this new genus are isolated and studied we shall refer to this culture as *Micropolyspora* sp. no. 1085.

METHODS

Isolation. Specimens of sputa taken in routine examination for *Mycobacterium tuberculosis* var. *hominis* were treated with 4% (w/v) NaOH and shaken on a mechanical shaker for 10 min. After neutralization with 2N-hydrochloric acid, the suspensions were centrifuged and the sediment used to inoculate two tubes of a Jensen-Holm modification of Lowenstein medium (*American Public Health Association*, 1950) and two tubes of penicillin blood agar (Tarshis, 1953). The tubes were incubated at 37°, horizontally for the first 72 hr. and then upright. They were examined weekly after the first 2 weeks.

Stains. Acid-fast staining was essentially as described on page 229 of Staining Procedures used by the Biological Stain Commission (Conn, Darrow & Emmel, 1960) except that one drop of Tergitol No. 7 (Carbide & Carbon Chemical Co., 30 East 42nd St., New York, N.Y.) was added to 30–40 ml. of carbol fuchsin just before staining. The Gram stains were done by covering smears for 1 min. with a 1% (w/v) aqueous solution of crystal violet and adding simultaneously five drops of 5% (w/v) aqueous sodium bicarbonate. After drainage, the slides were covered for 1 min. with an aqueous solution containing 2% (w/v) potassium iodide and 1% (w/v) mercury potassium iodide. After washing with water, the smears were decolorized with acetone, rinsed with water and stained for one minute with a 0.1% (w/v) aqueous solution of safranin Y.

Media. The composition of all media not listed here, and for which there are no references given in the text, will be found in the appendix of Waksman (1950) or in the *Difco Manual* (1953).

NZ-amine glycerol agar. NZ-amine A (casein hydrolysate; Sheffield Farms; 1267 6th Ave., New York), 5 g.; beef extract, 1 g.; glycerol, 70 ml.; agar, 15 g.; tap water, 1 l.; pH 6.5–7.0.

Nutrient agar with glucose. Nutrient agar with 1% (w/v) glucose.

Nutrient agar with glycerol. Nutrient agar with 70 ml. glycerol/l.

Soil extract glycerol agar. Peptone, 5 g.; beef extract, 3 g.; agar, 15 g.; soil extract, 250 ml.; tap water to 1 l.; pH 6.5–7.0. Soil extract was prepared by autoclaving 1 kg. air-dried garden soil with 2.4 l. tap water for 1 hr. at 121°, filtering through paper and autoclaving for storage.

Defined medium. Glycerol, 70 ml.; L-glutamic acid, 1.5 g.; L-arginine, 1.0 g.; K₂HPO₄, 1.0 g.; MgSO₄·7H₂O, 100 mg.; CaCl₂, 10 mg.; ZnSO₄, 10 mg.; Fe₂(SO₄)₃·9H₂O, 10 mg.; agar, 15 g.; distilled water, 1 l.; pH 6.5 to 7.0.

Photographs. Microphotographs were taken through a trinocular American Optical 'Microstar' Microscope with a 35 mm. photomicrography attachment. Kodak High Contrast Copy film was used. All microphotographs except Pl. 1, fig. 8, are of undisturbed plate cultures. Microphotographs were taken *in situ* through $\times 40$ or $\times 57$ achromatic objectives. The microscope was equipped with a long focus condenser which gave adequate lighting even through thick layers of agar. Plate 1, fig. 8, was taken through a $\times 93$ fluorite objective.

Electron photomicrographs (Pl. 1, figs. 6, 7) were taken with an RCA EMU-1 electron microscope modified with Canaleco equipment. Collodion films mounted on grids were touched to the surface of 5-day cultures grown at 37° on N-Z amine glycerol agar, portions of the mycelium becoming attached to the collodion membrane. The materials were not shadowed.

Examination of spore germination. Agar blocks from well-sporulating N-Z amine glycerol agar plate cultures were cut out aseptically and placed on sterile glass slides on the stage of a microscope. Spores were teased off the mycelium and spread over sterile portions of the agar blocks with a $5\ \mu$ sterile glass microhook, manoeuvred with a Cailloux micromanipulator. Slides were incubated in a humid chamber at 37° and examined periodically under the microscope. The number of germ tubes was counted after incubation for 18 hr.

RESULTS

Description of Micropolyspora Lechevalier, Solotorovsky and McDurmont, gen. nov.

Morphology. Fine mycelium (about $1\ \mu$ in diam.) which is differentiated into: (1) a substrate (primary) mycelium which grows into and forms a compact layer on top of agar media; (2) an aerial (secondary) mycelium which arises from the substrate mycelium and grows in the air away from the agar surface. Both the substrate hyphae and the aerial hyphae bear chains of conidia which are produced either directly on the mycelium or on sporophores which branch from the mycelium. Three strains were isolated: no. 1084, 1085 and 1086. The type species is based on the properties of no. 1084 and 1086. Isolate 1085, which is morphologically similar, has different cultural properties as indicated below.

Type species, Micropolyspora brevicatena, Lechevalier, Solotorovsky and McDurmont, sp. nov.

Substrate mycelium. About $1\ \mu$ in diameter. Filaments long, branching, penetrating the agar medium and forming compact colonies which are at first whitish on most media and become yellowish. Single spores or short chains of spores (2 to 10) are formed in the agar (Pl. 1, fig. 3) and on the surface of the agar where they are most easily observed (Pl. 1, figs. 1, 2).

Aerial mycelium. About $1\ \mu$ in diameter. Long branching hyphae, not abundant on most media. Short chains of spores formed (Pl. 1, figs. 4, 5). Often aerial hyphae will bend back in the agar after having grown in the air, forming solon-like structures (Pl. 1, fig. 9). Aerial hyphae will often aggregate to form long multi-filamentous strands which may link adjoining colonies. The tip of an aerial hypha, in young growing cultures, often curls up into a globose structure which is formed by the

tightly coiled filament (Pl. 1, fig. 4). These bodies have been observed to uncoil suddenly, with the resulting formation of a stolon. This sudden uncoiling was reminiscent of the casting of a fisherman's line.

Dome-shaped bodies. Hyphae often aggregate in a tough matty pseudo-tissue, which eventually forms a dome on the surface of the agar. Cutting through the cover of the dome reveals a hyaline gel which is rich in spores.

Spores. Spherical to oblong, sometimes pyriform, about $1.5\ \mu$ in diameter. Spores are borne terminally on sporophores (Pl. 1, fig. 7) which are sometimes branched (Pl. 1, fig. 6), as well as at the tip of main hyphae. They are also borne sessile on the side of hyphae. Spores are formed singly or in short chains (2 to 10 spores) and are most abundant and most easily observed on the surface of the agar. Isolate 1086 forms straight chains of spores, whereas the chains are slightly coiled or wavy in isolate 1084 (Pl. 1 figs. 1, 2). Spores are easily separated one from the other, but often stay and germinate in pairs. During germination, one to three germ tubes are formed. An incubation period of 5 to 7 days at 37° was optimal for the observation of the spores on the NZ-amine glycerol medium. The surface of the spores is very slightly warty (Pl. 1, figs. 6, 7).

Spore formation. A bud on a hypha grows to form a small branch, near the tip of which one septum appears; while the branch is still growing, a second septum is formed, then a third, etc. (Pl. 1, figs. 10, 11). The cells which have been walled off swell slightly, so that the spores are of a somewhat larger diameter than the hyphae, as indicated above (Pl. 1, figs. 3, 6, 7, 8). The mode of formation of the conidia is typically fungal in nature and is of the type illustrated in Fig. 251A of Langeron & Vanbreuseghem (1952).

Fragmentation. Even though fragmentation is not apparent during the *in situ* examination of the mycelium, either with bright field or phase contrast microscopy, the mycelium is easily separated into numerous fragments in the process of making smears.

Diffusible pigments. Isolate 1084 forms a light brown pigment after prolonged incubation (2 weeks) on certain media, such as NZ-amine glycerol agar. Isolates 1085 and 1086, in our studies, did not form any diffusible pigment.

Appearance on various media. All cultures were incubated for 2 weeks at 37° . No growth took place on the following media: starch agar A, starch agar B, glucose asparagine agar, Czapek agar. In addition, no growth occurred in nitrate broth, carbon-free cellulose medium, or oatmeal agar (Lechevalier & Lechevalier, 1957).

Yeast extract agar. Isolate 1084: thin growth with powdery surface; pale orange (Séguy 200). Isolates 1085 and 1086: thin growth with powdery surface; pale orange-yellow (Séguy 250).

Rice-extract agar and Pabulum-extract agar (Lechevalier & Lechevalier, 1957). Isolates 1084 and 1086: very little growth, powdery white. Isolate 1085: powdery white with a few light orange spots.

Potato plug. Isolate 1084: thin powdery growth, centre pale mauve (Séguy 239), edge pale orange-mauve (Séguy 180). Isolates 1085 and 1086: scarce thin powdery growth, pale mauve (Séguy 5).

NZ-amine glycerol agar. Isolate 1084: abundant, slightly wrinkled growth, pale orange (Séguy 200). Isolates 1085 and 1086: same as isolate 1084; pale orange-yellow (Séguy 250).

Defined medium. Isolate 1084: growth less abundant than on NZ-amine glycerol agar, pale orange (Séguy 199), white powdery surface. Isolate 1086: same, as 1084 pale orange-yellow (Séguy 250). Isolate 1085: no growth.

Nutrient agar. Isolate 1084: scarce, thin, buff growth. Isolate 1086: scarce, thin colourless growth. Isolate 1085: scarce, thin white growth.

Nutrient agar with glucose. Isolate 1084: powdery growth, thicker than on yeast extract agar, pale orange (Séguy 190). Isolate 1086: growth pale orange (Séguy 190) with a powdery white surface. Isolate 1085: thin powdery growth, pale orange-yellow (Séguy 250).

Nutrient agar with glycerol. Isolates 1084 and 1086: thick, shallowly wrinkled growth, pale orange-yellow (Séguy 249). Isolate 1085: thin powdery growth, pale orange-yellow (Séguy 250).

Litmus milk. Isolate 1084: slight powdery white pellicle, no effect on milk. Isolates 1085 and 1086: no growth.

Effect of pH and temperature on growth. Yeast-extract agar slopes were prepared at four different pH values (5.1, 6.3, 7.4, 8.4), inoculated with isolates 1084, 1085 and 1086, and incubated at 28°, 36° and 50°. Growth was best at pH 6.3 and 36°. No growth took place on any medium at 50°. No growth took place at pH 5.1 at any temperature.

Oxygen requirement. No growth took place on soil extract glycerol agar under a nitrogen atmosphere, or when the pyrogallol method (Fred & Waksman, 1928) of removing oxygen was used. Control aerobic cultures grew abundantly.

Defined medium for growth. In the presence of glucose, sucrose or glycerol, the following inorganic sources of nitrogen did not permit growth: NH_4NO_3 , NaNO_3 , $(\text{NH}_4)_2\text{SO}_4$. In presence of glycerol, DL-asparagine and DL-aspartic acid hardly permitted any growth, but L-glutamic acid was a good source of nitrogen for growth, even though sporulation was not abundant. Sporulation of isolates 1084 and 1086 was stimulated by L-arginine, which by itself was not satisfactory for growth and which prevented the growth of isolate 1085 in presence of L-glutamic acid. Good growth and sporulation of isolates 1084 and 1086 were obtained when magnesium sulphate, potassium phosphate, and calcium chloride were added to the L-arginine + L-glutamic acid + glycerol mixture. The addition of iron and zinc was not essential in this medium, which was obviously grossly contaminated with trace elements (see composition of the medium under METHODS).

Staining properties. The staining properties did not vary with the age of the culture (between 2 weeks and 2 months), but there was variation from one medium to another. On Lowenstein's and penicillin blood agar about 75 % of the organisms on smears were acid-fast, whereas on Sabouraud glucose and yeast-extract agar about 30 %. Less variation was observed with the Gram reaction, both organisms being about 50 % Gram-positive on all media.

Antibiotic properties. No activity by cross-streak test (Waksman, 1950) on yeast-extract glucose agar against *Aspergillus niger* 13, *Mucor rouxii* 80, *Candida albicans* 204, *Saccharomyces cerevisiae* 216, *Bacillus cereus* 8, *Escherichia coli* 54, or *Sarcina lutea* 14.

Sensitivity to antibiotics and antimicrobial agents. On yeast-extract agar, isolates 1084, 1085 and 1086 grew in the presence of pure candidin, 80 % pure candicidin, and pure cycloheximide at 100 µg./ml. Streptomycin at 100 µg./ml. inhibited

completely the growth of these isolates; chloramphenicol or neomycin at 5 $\mu\text{g./ml.}$ had the same effect. Neomycin at 10 $\mu\text{g./ml.}$ sterilized cultures of these isolates in 18 hr. at room temperature. The other antibiotics listed above had no microbicidal effect. On nutrient agar with glucose, the growth of the two cultures (no. 1804, 1806) was inhibited by isoniazid at 10 $\mu\text{g./ml.}$ and by neomycin at 1 $\mu\text{g./ml.}$

Source. The two strains of *Micropolyspora brevicatena* were isolated from the sputa of two adult white males who had been treated for tuberculosis, but who did not have active tuberculosis at the time the sputa were collected. Isolate 1085 was isolated from the sputum of a white adult female who had no active tuberculosis but whose husband had.

Pathogenicity. Intravenous and subcutaneous injections with heavy suspensions of isolates 1084 and 1085 in white mice produced no apparent ill effect. After 15 days, the animals were sacrificed and examined for evidence of pathological changes. Spleens in the inoculated animals were slightly larger than those of the controls. All other organs appeared normal. Microscopic examination of the organs of the animals did not reveal any specific lesions. Guinea-pigs injected intraperitoneally with heavy suspensions of isolate 1084 were unaffected as far as could be seen from the examination of the growth curves over a period of 30 days and gross examination of organs 30 days after injection of the culture suspensions.

DISCUSSION

As pointed out previously, in Waksman's classification of actinomycetes the basic difference between the two families Actinomycetaceae and Streptomycetaceae is based on the assumption that the substrate mycelium of members of the Actinomycetaceae fragments into bacillary or coccoid elements, whereas the substrate mycelium of the members of Streptomycetaceae remains unfragmented. It is very doubtful whether such a system is sound, since variation in fragmentation can be observed between isolates obtained from the same strain of organisms presently called *Nocardia* and *Streptomyces*.

The fact that the distinction between the two families is unsound is further demonstrated by the organism here described. *Micropolyspora* fragments like Actinomycetaceae, and sporulates like Streptomycetaceae by forming chains of conidia on aerial hyphae. In addition it has a novel mode of sporulation, namely, the formation of chains of conidia on the substrate mycelium. These conidia are located in and on agar media. It would be most unwise to solve the dilemma by creating a new family to accommodate the genus *Micropolyspora*; instead, it is suggested that the name Streptomycetaceae be dropped and that the family Actinomycetaceae be enlarged as follows.

Family Actinomycetaceae Buchanan, 1918: Branching hyphae about 1 μ in diameter form a substrate mycelium composed of hyphae, growing in and on agar media and an aerial mycelium formed of hyphae growing away from agar media. The aerial mycelium is sometimes lacking. The mycelium may or may not fragment into short segments which are either coccoid or rod-shaped. Mycelium may form single conidia or chains of conidia. Spores not formed in sporangia.

The following key to the genera of the Actinomycetaceae is proposed:

I. Anaerobic, or microaerophilic, organisms forming no conidia

1. *Actinomyces* Harz

II. Aerobic organisms. Might form conidia singly or in chains.

- A. Conidia formed singly
 - 1. No aerial mycelium
 - 2. Aerial mycelium formed
 - B. Conidia formed in longitudinal pairs on the aerial mycelium
 - C. Conidia formed singly and in chains on the substrate and on the aerial mycelium
 - D. Conidia when formed are in chains on the aerial mycelium only
- 2. *Micromonospora* Ørskov
 - 3. *Thermoactinomyces* Tsiklinsky
 - 4. *Waksmania* Lechevalier & Lechevalier
 - 5. *Micropolyspora* Lechevalier, Solotorovsky & McDurmont
 - 6. *Nocardia* Trevisan
 - 7. *Streptomyces* Waksman & Henrici

Fragmentation should not be confused with septation. Septa can be observed in the mycelium of some actinomycetes which do not have any tendency to fragment.

In the proposed key, attention has been paid mainly to morphological differences based on the types of conidia formed. It seems hardly proper to put morphologically similar organisms in different genera because one organism is mesophilic and the other thermophilic.

The distinction between *Nocardia* and *Streptomyces* is simple in certain cases. A typical *Streptomyces* sp. which formed a non-segmenting substrate mycelium and a well-developed aerial mycelium bearing chains of conidia is easily distinguished from an organism which forms a fragmenting substrate mycelium with poorly developed aerial hyphae which do not bear any conidia. Frequently, however, the distinction is hard to make, as shown by some recently published papers (Gordon & Mihm, 1958, 1961; Bradley, 1959).

In any event, *Micropolyspora brevicatena* represents a novel morphological type easily distinguishable from the previously described types that we have found in the literature and from those that we have studied up to now. One should note, however, that a thermophilic organism was described by Henssen (1957), under the name *Pseudonocardia thermophila*, which presumably forms chains of spores both on the substrate and aerial mycelium. The substrate mycelium of *Pseudonocardia* is septate and the chains of spores of this thermophilic organism seem to be fragments of the mycelium, quite different from the *Waksmania*-like conidia of *Micropolyspora*. Henssen's *Pseudonocardia thermophila*, judged on the basis of her description and photographs, seems to be a facultatively thermophilic *Nocardia*. We have examined two strains of *Pseudonocardia thermophila* (nos. 1179 and 1180) originally isolated by Henssen. We were unable to observe chains of conidia on the substrate mycelium.

A study of the ecology of strains of *Micropolyspora* would be of great interest. Such organisms have not been observed in soil. One might wonder if such organisms are to be found in healthy humans and animals, or, strangely, only in patients who have recovered from tuberculosis.

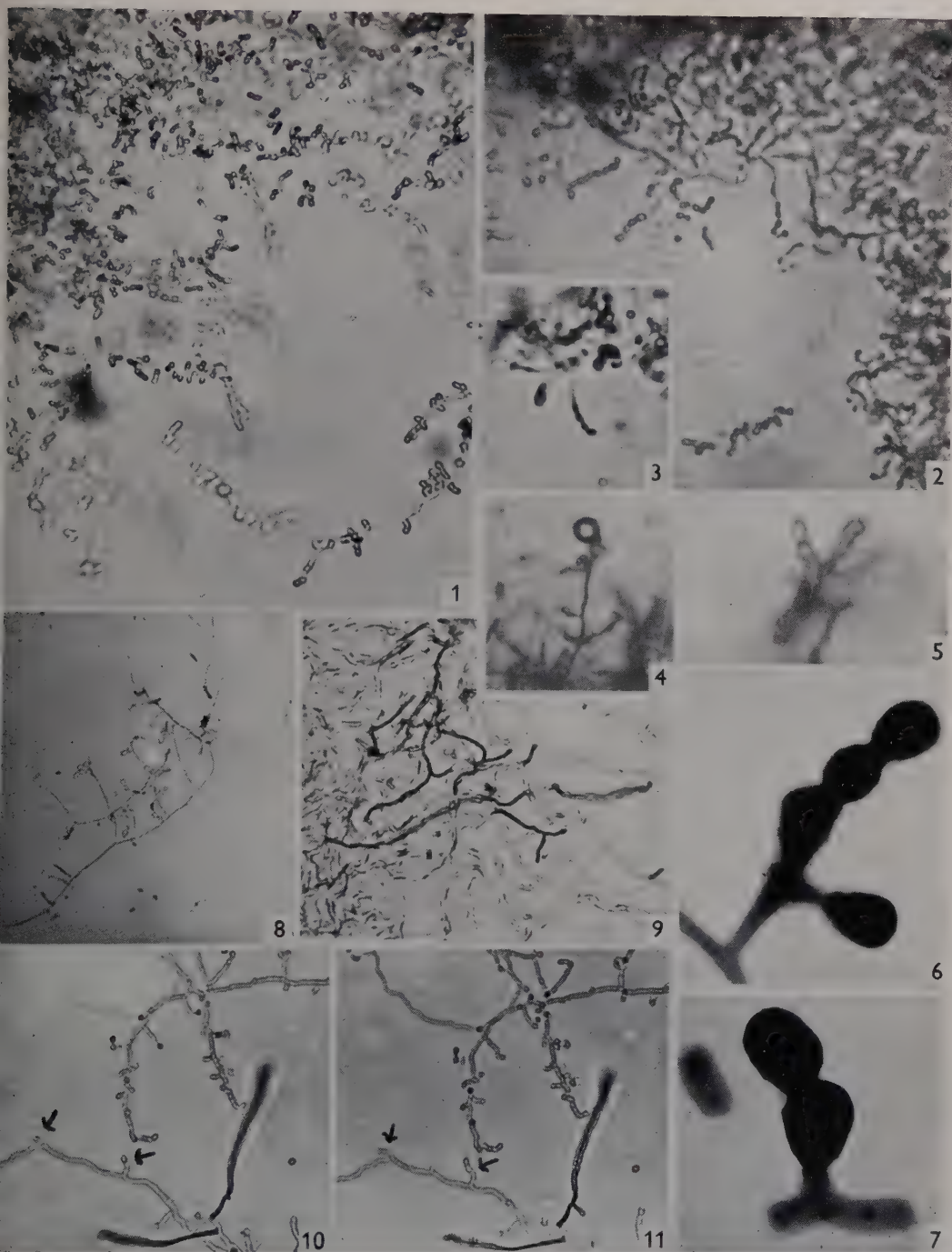
The authors wish to acknowledge the valuable advice and suggestions of Dr Ruth E. Gordon, and the technical assistance of Mrs Eva M. Fekete. The authors also wish to thank Miss Pauline E. Holbert for the electron micrographs. This work was supported in part by grant G-9694 from the National Science Foundation.

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EXPLANATION OF PLATE 1

- Fig. 1. Isolate 1086; chains of spores on the surface of the agar (NZ amine glycerol agar, 6-day culture). $\times 660$.
- Fig. 2. Isolate 1804; chains of spores on the surface of the agar (yeast-extract agar, 3-week culture). $\times 730$.
- Fig. 3. Isolate 1084; chain of spores deep in the agar (soil-extract glycerol agar, 20-day culture). $\times 670$.
- Fig. 4. Isolate 1086; chains of spores and globose structure on aerial hyphae (defined medium, 6-day culture). $\times 670$.
- Fig. 5. Isolate 1084; chains of spores on aerial hyphae (soil-extract glycerol agar, 20-day culture). $\times 2000$.
- Fig. 6. Isolate 1086; electron microphotograph of a chain of spores, with a branched sporophore. $\times 10,000$.
- Fig. 7. Isolate 1086; electron micrograph of a chain of spores. $\times 10,000$.
- Fig. 8. Isolate 1085; smear showing chains of spores. Gram stain. $\times 600$.
- Fig. 9. Isolate 1086; stolon-like aerial hyphae (NZ-amine glycerol agar, 2-day culture). $\times 800$.
- Fig. 10. Isolate 1084; 4-day culture on nutrient agar with glycerol. $\times 800$.
- Fig. 11. Same culture 7 hr. later. Note formation of spores. $\times 800$.



H. A. LECHEVALIER, M. SOLOTOROVSKY AND C. I. McDURMONT

(Facing p. 18)

Recombination in *Actinomyces aureofaciens*

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SUMMARY

Biochemical mutants of *Actinomyces aureofaciens* were obtained after treatment with ultraviolet irradiation or ethyleneimine. A specificity of the mutational process in a series of *A. aureofaciens* strains was observed, revealing itself mainly in formation of arginine-requiring mutants. Prototroph formation was shown to result from crosses of *A. aureofaciens* biochemical mutants with either the same or different amino acid requirements. Colonial morphology and antibacterial activity of some prototrophs were studied.

INTRODUCTION

The possibility of obtaining hybrids or heterozygous diploids in various species of imperfect fungi was shown for the first time by Pontecorvo (1956) and others. This was extended to antibiotic-producing actinomycetes by Sermonti & Spada-Sermonti (1955, 1956) and by Sermonti (1957). Later the methods developed by these authors were used by Alikhanian & Mindlin (1957) for obtaining recombinants in *Actinomyces rimosus*. The aim of this investigation was to study the possibility of hybridization in *A. aureofaciens* so as to use it for the purposes of selection.

METHODS

Biochemical mutants were obtained after ultraviolet (u.v.) irradiation and ethyleneimine treatment of *Actinomyces aureofaciens*. At the very beginning of our studies we found that this organism differed greatly from other actinomycetes in the extreme specificity of the mutational process in a number of strains, such as 536, II, Bd, BMK, B-16. These strains of *A. aureofaciens* differed from each other in their origin, colonial morphology and antibiotic-producing properties. The biochemical mutants were isolated on the following media: corn steep liquor (N12) medium, containing (w/v) 0.5% corn steep liquor, 0.4% $(\text{NH}_4)_2\text{HPO}_4$, 0.2% KH_2PO_4 , 0.29% MgSO_4 , 0.1% CaCO_3 , 2% starch, 2.0% agar; and two modifications of this medium, one with the addition of yeast extract and another with the addition of yeast extract and casein hydrolysate. The data on selection of biochemical mutants in *A. aureofaciens* are presented in Table 1.

As is evident from Table 1 our data do not conform to the regularities of biochemical mutant formation described in the literature in that from all strains, independent of the nutrient medium used, the occurrence of arginine-requiring (*arg*) mutants only was in general observed (99 cases), and only in two cases other amino acid requiring mutants (histidine (*his*) and isoleucine+valine (*isl+val*)) were detected.

The occurrence of arginine-requiring mutants in almost all cases could not but direct our attention to study hybridization in *Actinomyces aureofaciens*. The prototrophs were obtained by the method described by Alikhanian & Mindlin (1957).

Table 1. *The frequency of isolation of biochemical mutants from different strains of Actinomyces aureofaciens treated with various mutagenic factors*

Strain	Mutagenic factor	Medium	No. of biochemical mutants	Nutritional requirements	No. of isolates examined	
BMK	u.v. radiation	N 12	21	Arginine	1300	
		N 12 + yeast extract	7	Arginine	—	
		N 12 + yeast extract and casein	5	Arginine	—	
	Ethyleneimine	N 12	0	—	1075	
		N 12 + yeast extract	0	—	—	
		N 12 + yeast extract and casein	0	—	—	
Bd	u.v. radiation	N 12	19	Arginine	1211	
		N 12 + yeast extract and casein	4	Arginine	—	
	Ethyleneimine	N 12	0	—	1200	
		N 12 + yeast extract	2	Arginine	—	
		N 12 + yeast extract and casein	1	Arginine	—	
	536	u.v. radiation	N 12	5	Isoleucine + valine	3319
				Arginine (4)	—	
N 12 + yeast extract			10	Arginine	—	
		N 12 + yeast extract and casein	0			
Ethyleneimine		N 12	4	Arginine	1273	
		N 12 + yeast extract	7	Arginine	—	
		N 12 + yeast extract and casein	1	Arginine	—	
B-16		u.v. radiation	N 12	6	Arginine	2006
			N 12 + yeast extract	3	Arginine	—
	N 12 + yeast extract and casein		4	Histidine (1)	—	
			Arginine (3)	—		
	Ethyleneimine	N 12	0	—	1740	
		N 12 + yeast extract	0	—	—	
		N 12 + yeast extract and casein	0	—	—	
	II	u.v. radiation	N 12	1	Arginine	3071
			N 12 + yeast extract	0	—	—
N 12 + yeast extract and casein			0	—	—	
Ethyleneimine		N 12	0	—	1600	
		N 12 + yeast extract	0	—	—	
		N 12 + yeast extract and casein	0	—	—	

RESULTS

It was first necessary to demonstrate the possibility of recombination in *Actinomyces aureofaciens* in order that crosses might be made between all arginine-requiring mutants to explain their genetic nature. For this purpose we used the isoleucine + valine-requiring mutant, the histidine-requiring mutant, and a number of arginine-requiring mutants. The data on the investigation of combinations in the three groups are presented in Table 2.

Table 2. *Biochemical mutant combinations and the frequency of prototroph formation from these combinations*

Combination group	Biochemical combination	Prototroph frequency (%)	Frequency biochemical mutant reversions
I	<i>arg-1</i> × (<i>isl</i> + <i>val</i>)	0.13	0
	<i>arg-2</i> × (<i>isl</i> + <i>val</i>)	0.06	0
	<i>arg-3</i> × (<i>isl</i> + <i>val</i>)	0.0009	0
	<i>arg-4</i> × (<i>isl</i> + <i>val</i>)	3.3	0
	<i>arg-5</i> × (<i>isl</i> + <i>val</i>)	0.0004	0
	<i>arg-6</i> × (<i>isl</i> + <i>val</i>)	0.13	0
	<i>arg-7</i> × (<i>isl</i> + <i>val</i>)	0.01	0
	<i>arg-8</i> × (<i>isl</i> + <i>val</i>)	0.03	0
	<i>arg-9</i> × (<i>isl</i> + <i>val</i>)	0.006	0
	<i>arg-10</i> × (<i>isl</i> + <i>val</i>)	0.0	0
	<i>arg-11</i> × (<i>isl</i> + <i>val</i>)	0.004	0
	<i>arg-12</i> × (<i>isl</i> + <i>val</i>)	0.02	0
	<i>arg-13</i> × (<i>isl</i> + <i>val</i>)	0.0025	0
	<i>arg-14</i> × (<i>isl</i> + <i>val</i>)	0.0003	0
II	<i>arg-2</i> × <i>his</i>	0.0015	0
	<i>arg-7</i> × <i>his</i>	0.0007	0
III	<i>his</i> × (<i>isl</i> + <i>val</i>)	0.9	0

Biochemical mutants *arg-1* to *arg-5* were derived from strain Bd; *arg-6* to *arg-14* from strain BMK; (*isl* + *val*) from strain 536; *his* from strain B-16.

It should be pointed out that these crosses gave rise to a comparatively high frequency of prototrophs, reaching 3.3% in some combinations. This was higher than the frequencies observed in crosses of *Actinomyces rimosus*.

The prototrophs, obtained from the various combinations, independent of the starting strains, belonged morphologically to the classical wild-type strain of *A. aureofaciens* as for example strain 536 type. Such prototrophs formed flat colonies of mouse-grey or dark-grey coloration with aerial mycelium and buff-yellow substrate mycelium. Some prototrophs were characterized by a different rate of sporulation but this cannot serve as a distinguishing feature. Prototrophs from combination *arg-7* × *his* may be regarded as an exception, since they were characterized by dingy-beige coloured spores and low rate of sporulation.

In contrast, the biochemical mutants from which the prototrophs were derived differed greatly in colonial morphology from the wild-type strains. Thus, all arginine-requiring mutants were characterized by flat colonies, not highly folded or having radial lines, asporogenic, with no aerial mycelium or specific pigment (Plate 1). The histidine-requiring mutant formed light-grey, flat, compact colonies, elevated above

the agar surface and characterized by a low rate of sporulation and limited growth. The mutant requiring isoleucine and valine differed from the wild-type strains only by the production of colonies with poor sporulation and a broad asporogenic periphery.

Segregation patterns of prototrophs

The prototrophic recombinants gave rise to various types of segregants with differing frequencies. In order to study in detail the various segregation patterns we used prototrophs from eleven combinations, eight combinations of group I (*arg-1* to

Table 3. *Distribution of prototrophs from combinations according to the segregation types*

Combination group	Biochemical mutant combination	Prototrophs studied	Segregation type
I	<i>arg-1</i> × (<i>isl</i> + <i>val</i>)	No. 1	I
		No. 2	I
		No. 3	I
		No. 4	II
		No. 5	VI
	<i>arg-2</i> × (<i>isl</i> + <i>val</i>)	No. 1	I
		No. 2	I
		No. 3	I
		No. 4	IV
		No. 5	VI
		No. 6	VI
		No. 7	VI
	<i>arg-3</i> × (<i>isl</i> + <i>val</i>)	No. 1	I
		No. 2	II
		No. 3	III
	<i>arg-4</i> × (<i>isl</i> + <i>val</i>)	No. 1	I
		No. 2	I
	<i>arg-5</i> × (<i>isl</i> + <i>val</i>)	No. 1	I
		No. 2	I
		No. 3	II
	<i>arg-6</i> × (<i>isl</i> + <i>val</i>)	No. 1	I
		No. 2	I
		No. 3	I
		No. 4	IV
		No. 5	IV
	<i>arg-7</i> × (<i>isl</i> + <i>val</i>)	No. 1	I
		No. 2	I
		No. 3	V
	<i>arg-8</i> × (<i>isl</i> + <i>val</i>)	No. 1	IV
II	<i>arg-2</i> × <i>his</i>	No. 1	I
		No. 2	II
		No. 3	VI
		No. 4	VI
		No. 5	VI
	<i>arg-7</i> × <i>his</i>	No. 1	I
		No. 2	IV
III	<i>his</i> × (<i>isl</i> + <i>val</i>)	No. 1	III
		No. 2	VI
		No. 3	VI
		No. 4	VI
		No. 5	VI

arg-8 × (*isl* + *val*) and all combinations of group II (*arg-2* × *his*; *arg-7* × *his*) and group III (*his* × (*isl* + *val*)). As a result of this study the segregation patterns which are summarized in Table 3 were observed.

In all, the behaviour of 41 prototrophs from eleven combinations were studied. As far as their segregation pattern is concerned, six types were observed which were produced to a varying extent from each combination.

Segregation of type I. When plated out, the prototrophs of this type resembled the starting prototrophs, but segregated one of the starting biochemical mutants, in this instance the arginine-requiring mutant, but only in very small amounts (0.02–0.7 %).

The arginine-requiring segregant in its turn segregated in the first and sometimes in the second generation a few prototrophic colonies (0.5 %), morphologically similar to the arginine-requiring mutant. Further segregation of such colonies was not observed. It should be noted that the arginine-requiring mutants never segregated prototrophic or other auxotrophic forms. Neither were reverse spontaneous mutations observed.

Segregation of type II. A considerably smaller number of the prototrophs segregated the other parental form, that is the form similar to the isoleucine + valine-requiring mutant. The amount of such segregants was 1.5 to 0.14 %.

The isoleucine + valine-requiring segregant in further generations segregated no new forms.

Segregation of type III. A single prototroph (one prototroph among 41 prototrophs studied) which segregated both parental biochemical mutants (1.4 to 0.1 %).

Segregation of type IV. In this type of segregation, the prototroph segregated morphologically changed prototrophs. The number of such prototrophs was not high (0.15 %). In most cases, asporogenic forms similar to the arginine-requiring mutant were observed. Sometimes solid colonies with black substrate mycelium and various degrees of sporulation were encountered.

Table 4. *Quantitative distribution of prototrophs according to the segregation types*

Segregation type	No. of prototrophs	Segregation type	No. of prototrophs
I	18	IV	5
II	4	V	1
III	2	VI	11

Segregation of type V. In the first generation prototrophs belonging to this type segregated only morphologically-changed prototrophs, while in the second generation segregation of arginine-requiring variants was also observed.

Segregation of type VI. Prototrophs of type VI proved to be stable and segregated no new forms in a number of generations. The quantitative distribution of all 41 prototrophs according to these six types of segregation is presented in Table 4. As is evident from Table 4, the prototrophs belonging to type I, as well as stable prototrophs were the most common.

It should be pointed out that prototrophs segregating according to different types were detected from the same combination. Thus, five prototrophs obtained from the combination *arg-7* × (*isl* + *val*) segregated according to three segregation types. Three of them belonged to type I, one to type II, and one to type VI.

Antibacterial activity of prototrophs

110 prototrophic colonies were studied with respect to the amount of chlortetracycline produced. The antibacterial activity was estimated on submerged cultures by the colorimetric assay method after acid hydrolysis of the culture fluid. Biochemical mutants and the wild-type strains, from which they were derived, served as the controls. The activity of the biochemical mutants was never greater than 100 $\mu\text{g./ml.}$, the activity of the arginine-requiring mutants in most cases being equal to zero, while the activity of the wild-type strains was 850–1000 $\mu\text{g./ml.}$ The activity of all prototrophs was many times higher than that of the biochemical mutants and reached the activity of the starting strains. Sometimes the activity of all the prototrophs from one combination was higher than that of the starting strains by 5–20 %. Examples of the antibacterial activity of prototrophs from three combinations ($\text{arg-1} \times (\text{isl} + \text{val})$; $\text{arg-4} \times (\text{isl} + \text{val})$; $\text{arg-3} \times (\text{isl} + \text{val})$) are presented in Table 5 and the corresponding diagram (Fig. 1).

The activity of prototrophs from combinations $\text{arg-2} \times (\text{isl} + \text{val})$; $\text{arg-7} \times (\text{isl} + \text{val})$; $\text{arg-8} \times (\text{isl} + \text{val})$; $\text{arg-5} \times (\text{isl} + \text{val})$, $\text{arg-7} \times \text{his}$; $\text{his} \times (\text{isl} + \text{val})$ was no higher than that of any of the wild-type strains similar to the activity of prototrophs from combination $\text{arg-4} \times (\text{isl} + \text{val})$. Some prototrophs from combinations $\text{arg-6} \times (\text{isl} + \text{val})$ and $\text{arg-2} \times (\text{isl} + \text{val})$ were superior with respect to their activity as compared with the wild-type strains, resembling those prototrophs from combination $\text{arg-1} \times (\text{isl} + \text{val})$. Combination $\text{arg-3} \times (\text{isl} + \text{val})$ was the only one among the eleven combinations studied, which produced prototrophs always superior in their chlortetracycline activity to the wild-type strains.

Table 5. *Chlortetracycline activity of the wild-type strains, biochemical mutants and prototrophs from combinations $\text{arg-4} \times (\text{isl} + \text{val})$, $\text{arg-1} \times (\text{isl} + \text{val})$ and $\text{arg-3} \times (\text{isl} + \text{val})$*

Strain	Activity ($\mu\text{g./ml.}$)	Strain	Activity ($\mu\text{g./ml.}$)
Bd	990	<i>arg-1</i>	0
536	850	<i>arg-3</i>	0
<i>arg-4</i>	0	(<i>isl</i> + <i>val</i>)	50
Prototrophs from combination $\text{arg-4} \times (\text{isl} + \text{val})$			
No. 1	351	No. 6	316
No. 2	814	No. 7	582
No. 3	732	No. 8	632
No. 4	707	No. 9	420
No. 5	802	No. 10	772
Prototrophs from combination $\text{arg-1} \times (\text{isl} + \text{val})$			
No. 4	678	No. 9	1086
No. 5	256	No. 10	1143
No. 6	884	No. 11	1062
No. 7	241	No. 15	326
No. 8	466	No. 17	391
Prototrophs from combination $\text{arg-3} \times (\text{isl} + \text{val})$			
No. 1	1199	No. 9	1288
No. 2	1038	No. 11	1223
No. 3	1152	No. 14	1080
No. 5	1288	No. 16	1109
No. 7	1203	No. 17	996

It is interesting to note that in the combinations $arg-4 \times (isl + val)$, $arg-1 \times (isl + val)$ and $arg-3 \times (isl + val)$, all of which produced prototrophs differing in antibacterial activity, one and the same isoleucine + valine requiring mutant, derived from strain 536, was used. As the other crossing component in these combinations, one of the arginine-requiring mutants, $arg-4$, $arg-3$ or $arg-1$ derived from strain Bd, was used. These mutants differed neither in colonial morphology, nor in antibacterial activity. In addition to the arginine deficiency, each of these mutants is likely to possess specific physiological properties which determine the difference of the

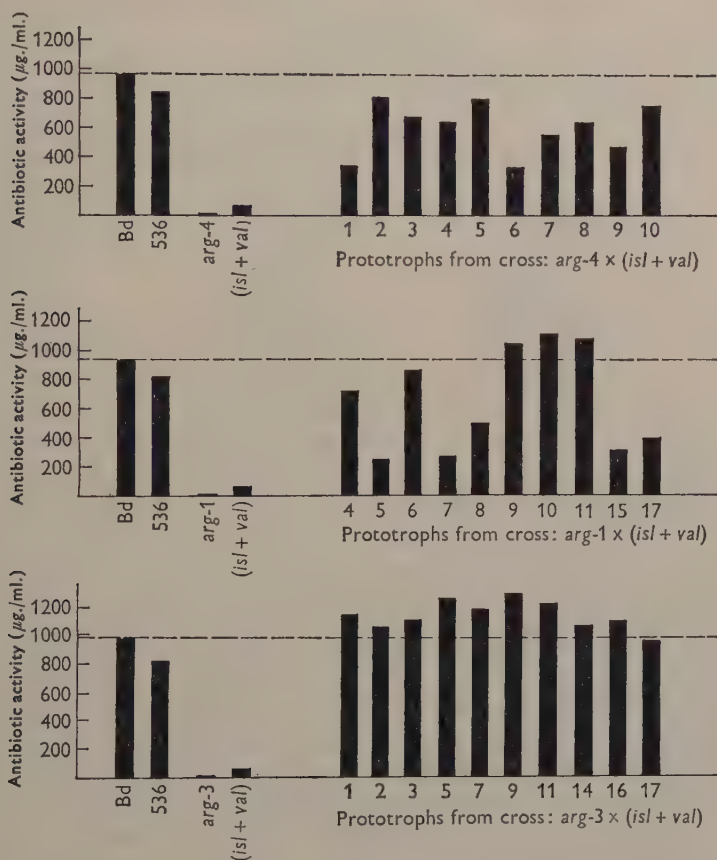


Fig. 1. Antibiotic (chlortetracycline) activity of the wild-type strains, biochemical mutants and prototrophs. The activity levels are presented in absolute values. The interrupted line indicates the highest activity level of the wild-type strains.

prototrophs from the above combinations with respect to their antibacterial activities.

One prototroph which proved to be the most active, that is prototroph no. 11 from combination $arg-3 \times (isl + val)$, was selected for studying the range of induced variation in comparison with the wild-type strains. Antibacterial activity of the prototrophic-segregants was also studied. Usually the activity of non-sporulating prototrophs similar in their morphological properties to the arginine-requiring

mutants was zero, while the activity of segregants, such as segregant no. 3 from combination *arg-7* × (*isl* + *val*) reached 300 µg./ml. After these experiments, demonstrating recombinant formation in *Actinomyces aureofaciens*, combinations within the arginine-requiring mutants were studied.

In these combinations 56 arginine-requiring mutants of the 99 mutants isolated were used. All these strains were very similar in colonial morphology. They were characterized by asporogenic flat colonies, not highly folded nor having radial lines, and with no aerial mycelium or specific pigment.

Table 6. *Combinations of arginine-requiring mutants from which prototrophs were obtained*

No reversions of arginine-requiring strains were obtained

Combination	Biochemical mutant combination*	No. of prototrophs obtained
No. 24	<i>arg-19</i> × <i>arg-20</i>	8
No. 25	<i>arg-21</i> × <i>arg-9</i>	2
No. 52	<i>arg-24</i> × <i>arg-23</i>	1
No. 1108	<i>arg-15</i> × <i>arg-1</i>	1
No. 1073	<i>arg-18</i> × <i>arg-3</i>	1
No. 1107	<i>arg-15</i> × <i>arg-16</i>	1
No. 1176	<i>arg-11</i> × <i>arg-17</i>	1
No. 958	<i>arg-11</i> × <i>arg-1</i>	2
No. 1167	<i>arg-7</i> × <i>arg-22</i>	1
No. 1155	<i>arg-23</i> × <i>arg-22</i>	2
No. 306	<i>arg-25</i> × <i>arg-23</i>	1

* Biochemical mutants *arg-7*; *arg-9*; *arg-11*; *arg-15*; *arg-18*; *arg-19*; *arg-20*; *arg-21*; *arg-23*; *arg-24* were derived from strain BMK; *arg-1*; *arg-3*; *arg-16*; *arg-17* and *arg-25* from strain Bd; *arg-22* from strain B-16.

The strains are very stable and segregate neither prototrophic, nor auxotrophic forms over a number of generations. An attempt was made to differentiate the mutants with respect to their requirements for intermediary products of arginine biosynthesis, that is for either glutamic acid, ornithine or citrulline; or according to an alternative way of synthesis, for urea or guanidine. It was found that all the arginine-requiring mutants isolated were similar in that the addition of any of the above intermediates to the medium did not restore the synthesis of arginine and permit growth. 1540 combinations of arginine-requiring mutants were studied. Prototrophs were obtained in eleven cases (Table 6). The frequency of prototrophs in these combinations is as low as 1 in 10⁷.

The prototrophs obtained differed in colonial morphology from one another and from strain 536 typical of *Actinomyces aureofaciens*. Most of them formed well sporulating colonies of white to light-grey coloration, with sporophores in the form of spirals with 0.5–1.0 µ coils, which is characteristic of *A. aureofaciens*. Some prototrophs formed asporogenic colonies, morphologically similar to those produced by arginine-requiring mutants (Plate 1).

The study of these prototrophs with respect to their segregation pattern showed that the majority of them were stable and segregated no new forms in a number of generations. Only one prototroph, that is no. 1073, segregated a small amount (0.3%) of non-sporulating prototrophs. Therefore we had no data, with respect to

direct segregation of the starting biochemical mutants, to indicate the deviation of these prototrophs by recombination. However, certain changes were found in the antibiotic-producing properties of these prototrophs as compared with the arginine requiring mutants (Table 7).

Table 7. *Antibacterial activity of the wild-type strains, arginine-requiring mutants, and prototrophs from combinations of these mutants*

Strain	Chlortetra- cycline activity ($\mu\text{g./ml.}$)	Strain	Chlortetra- cycline activity ($\mu\text{g./ml.}$)
Starting strains			
BMK	950	B-16	1600
Bd	990		
Arginine-requiring mutants			
<i>arg-24</i>	0	<i>arg-7</i>	20
<i>arg-23</i>	80	<i>arg-22</i>	0
<i>arg-15</i>	0	Prototrophs from	
<i>arg-1</i>	80	<i>arg-23</i> \times <i>arg-24</i>	0
<i>arg-18</i>	0	<i>arg-1</i> \times <i>arg-15</i>	0
<i>arg-3</i>	0	<i>arg-18</i> \times <i>arg-3</i>	0
<i>arg-11</i>	20	<i>arg-11</i> \times <i>arg-17</i>	0
<i>arg-17</i>	16	<i>arg-7</i> \times <i>arg-22</i>	0

DISCUSSION

As is evident from the data presented in Table 6, 16 arginine-requiring mutants out of 56, on crossing in definite combinations, produced the parental form, that is gave rise to prototrophs. It is possible to assume that they belong to different alleles, though probably not to different genes. All prototrophs from combinations of arginine-requiring mutants independent of the activity of the wild-type strains were unable to synthesize chlortetracycline. This was not observed in the other crossings of *Actinomyces aureofaciens* involving mutants having dissimilar biochemical requirements and many crossings of *A. rimosus*. When *A. aureofaciens* mutants with different nutritional requirements were crossed, even when the antibacterial activity of these mutants was either zero or as low as $50\mu\text{g./ml.}$, the prototrophs obtained were similar in their activity to the wild-type strains. On the basis of these data it is possible to assume that the arginine-producing locus is related to the chlortetracycline-producing locus. This assumption is confirmed by the specificity of biochemical mutant formation in *A. aureofaciens*, as well as by the fact that active hybrids are formed only in combinations, where one of the crossing components is a biochemical mutant requiring histidine or isoleucine + valine. It should be noted that the data presented are preliminary, and further studies on arginine-requiring mutants are needed.

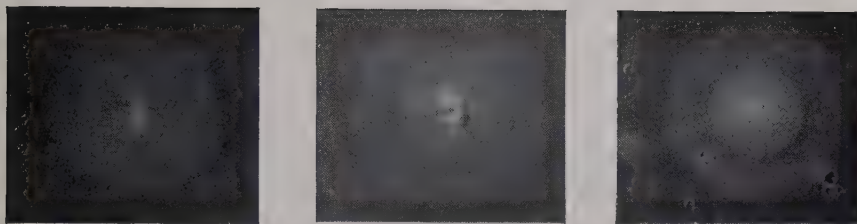
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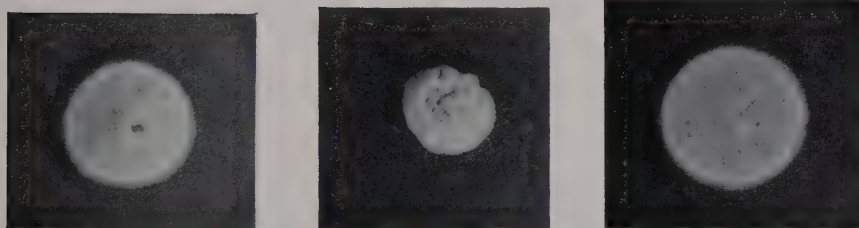
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EXPLANATION OF PLATE

Colonies of arginine-requiring mutants and of prototrophs obtained from combinations of arginine-requiring mutants. Grown on medium N12 for 8 days.



Arginine-requiring mutants



Prototrophs obtained from combinations of arginine-requiring mutants

The Habitat and Description of a New Genus of Sulphur Bacterium

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SUMMARY

A new species and genus of colourless sulphur bacteria, for which the name *Thiodendron mucosum* is proposed, is described from two habitats in Florida. Its morphology and physiology relate it to the Beggiatoaceae, but it is branching, non-filamentous and non-motile so it should not be included in that family and a new family should be created for it. Some characteristics of its environment are discussed and also the organisms common to such an environment. It is probable that the particular organisms are closely connected to the metabolism of sulphur, while their variety and biomass attribute an important role to sulphur in nature, especially in environments containing little or no oxygen.

INTRODUCTION

In January 1956 a collection of sulphur bacteria was made at Warm Mineral Spring near Venice in south-west Florida, U.S.A. One of these was considered to be new. Dr E. G. Pringsheim of the Botanische Austatem, The University of Göttingen, Germany, was visiting the laboratory at the time and he concurred in this view. Since that date the organism has been studied intensively, and has been found at only one other location, despite diligent search.

METHODS

The work reported here consisted of making field collections from sulphur springs and other environments, and studying the organisms in the laboratory under high magnifications. Physicochemical characteristics of the waters were also determined where possible, and some staining was done. The organisms stained very unevenly by Gram's method, overstained intensely with carbol-fuchsin and did not take up methylene blue. Apparently the large amount of jelly-like material interferes greatly with the stains used. Many laboratory culture methods were tried, but since none has been successful thus far, they are not described here.

The State of Florida, U.S.A., has seventeen first-magnitude springs (Ferguson, Lingham, Love & Vernon, 1947), as well as vast numbers of smaller ones. Many of these are sulphur springs, giving off H_2S and supporting white growths of sulphur bacteria, principally Beggiatoa and Thiothrix. These growths appear as a white coating on the spring bottom and sides and often extend far down the run. All the springs are constant in their characteristics, although differing sharply among themselves; for this reason Odum (1957) referred to them as excellent laboratories. Most

of the sulphur springs show a single dominant species of sulphur bacteria with others in smaller numbers. The white coating is due to the dominant organism. Table 1 lists the sulphur-accumulating bacteria belonging to the families Beggiatoaceae and Thiobacteriaceae which were abundant at some of the eleven stations studied.

Warm Mineral Spring is an exception to the single dominant condition because its huge population consists principally of six species of *Beggiatoa* and five species of *Thiothrix* and because dominance shifts. It is also the only spring whose salt content is so high; Table 2 gives its physicochemical characteristics. Its temperature gradient suggests that the water comes from Ocala limestone at a depth of about 800 ft., and Dr A. P. Brooks (private communication) suggested that the water is 50 % connate sea water and 50 % fresh water. There is virtually no fluctuation in any characteristic, except that of light, and this only to a depth of about 65 ft. The water is very clear in the early morning, but after sunrise it becomes cloudy because of a photochemical precipitation of sulphur. Sulphur granules are easily found free in the sand and water; this photochemical mechanism was not mentioned by Ivanov (1957) in addition to his account of the precipitation of sulphur by bacteria, although indeed he was working principally with sulphur in underground formations.

Table 1. Occurrence of colourless sulphur-accumulating bacteria at eleven locations

All these bacteria were identified during the years 1956-60 by direct microscopic examination, except *Thiobacillus denitrificans* which was cultured. The identification of *Thiobacterium bovista* might be questionable.

	Locations										
	1	2	3	4	5	6	7	8	9	10	11
1. Warm Mineral Spring, Fla. Half salt.											
2. Orange Springs, Fla. Fresh.											
3. Welaka Mud Springs, Fla. Fresh.											
4. Sarasota, Fla., Ringling Fountain. Fresh.											
5. Green Cove Springs, Fla. Fresh.											
6. Fort Myers, Fla. Fresh.											
7. Sanibel Island, Fla. Fresh.											
8. Hampton Springs, Fla. Fresh.											
9. Lake Alice, Gainesville, Fla. Fresh.											
10. Titusville, Fla., Inland Waterway. Salt.											
11. Woods Hole, Mass., Salt Marshes.											
	Location										
	1	2	3	4	5	6	7	8	9	10	11
<i>Achromatium oxaliferum</i>	X	X	.	X
<i>A. volutans</i>	X	X	X
<i>Beggiatoa alba</i>	X	X	X	X	X	.	X	X	X	X	X
<i>B. arachnoidea</i>	X	.	X	.	X	.	.	.	X	X	X
<i>B. gigantea</i>	X	X	X
<i>B. leptomitiformis</i>	X	.	X	.	X	.	X	X	X	.	X
<i>B. minima</i>	X	X	.	X
<i>B. mirabilis</i>	X	X	X
<i>Thiodendron mucosum</i> n.sp.	X	X	.
<i>Thiospirillopsis floridana</i>	X
<i>Thiothrix annulata</i>	X	.	X
<i>T. marina</i>	X	X	X
<i>T. nivea</i>	X	.	X	.	X	X	X	X	X	.	X
<i>T. tenuis</i>	X	.	X	X	X	.	.	X	X	.	.
<i>T. tenuissima</i>	X	.	X
<i>Macromonas bipunctata</i>	X	X	X
<i>M. mobilis</i>	X	.	X
<i>Thiobacillus denitrificans</i>	X	X	X	X	X	.	X
<i>Thiobacterium bovista?</i>	X	X
<i>Thiospira winogradskyi</i>	X	X	X
<i>T. bipunctata</i>	X	X	X	X
<i>Thiovulum majus</i>	X	X	.	X	X	X	X

X = present

Table 2. *Physicochemical characteristics of Warm Mineral Spring*

	Ferguson <i>et al.</i> (1947)	Morgan (1956)
Dissolved solids	17,812 p.p.m.	17,988 p.p.m.
Iron	0.12 p.p.m.	0.09 p.p.m.
Calcium	766 p.p.m.	596 p.p.m.
Magnesium	471 p.p.m.	567 p.p.m.
Silica (SiO ₂)	18.00 p.p.m.	23.80 p.p.m.
Sodium, potassium	5,124 p.p.m.	.
Chloride	9,350 p.p.m.	.
Bicarbonate (HCO ₃)	.	167 p.p.m.
Total hardness (CaCO ₃)	3,846 p.p.m.	.
Temperature	.	84° F. ± 2°
pH value	.	7.2 ± 0.2
Volatile solids (600° for 30 min.)	.	17.1 %
Nitrate	.	0.05 p.p.m.
Dissolved PO ₄	.	0.0016 p.p.m.
Total PO ₄	.	0.0037 p.p.m.
C.O.D. (dichromate)	.	813 p.p.m.
Dissolved oxygen*	.	0.0 p.p.m.
H ₂ S	.	0.162 p.p.m.
HS	.	0.078 p.p.m.
SO ₄	.	1704 p.p.m.

* Odum says that interference accounts for this value; if the interference is removed, a value of 1.0 is obtained.

RESULTS

Occurrence of the new bacterium

The first specimens of the new organism were found in January 1956. Collections of the dense growths of sulphur bacteria were brought to the laboratory for studies of the widths of the various types. Among these were arboroid colonies densely packed with what appeared to be sulphur granules. Most of these were attached to the blue-green alga *Lyngbya*. Some of the colonies had a spread of over 2 mm. They were examined alive, with a Zeiss Optovar microscope at magnifications of 100–1600 diameters. Some were set aside in small moist chambers (Petri dishes containing filter paper saturated with salt water) for subsequent examination. It was concluded that the new organisms represented a free-living sulphur bacterium of a new type. A second trip to the spring 3 weeks later yielded no specimens, but it has since been found many times in this spring, and as far as half a mile down the run. Many other sulphur springs in Florida have been investigated without finding the new organism nor was it found in H₂S-containing water at La Jolla, California, at Woods Hole, Massachusetts, in the desert around Las Vegas, Nevada, and in aquaria containing good natural growths of *Beggiatoaceae* in the Narragansett Marine Laboratory, Rhode Island. However, in February 1959 a heavy coating of sulphur bacteria was found in shallow water along the intracoastal waterway at Titusville, Florida. Examination revealed many colonies of the new bacterium, and it has been found at all subsequent visits to this location. These sulphur bacteria (*Beggiatoales*) were in a grossly polluted situation behind a crab-meat packing plant. There should be no pollution at all in Warm Mineral Spring (now being exploited as a spa), but it should be noted that the volatile solids are very high for such a situation.

Description of the organism

This description is based primarily upon microscopic examination of unkilld, unfixed material. Two small blobs of viscous grease were put on a slide, a drop of water containing the organism was added between the bits of grease, and a no. 1 thickness cover glass was added. This permitted brightfield and darkfield examination at magnifications up to 1600 diameters.

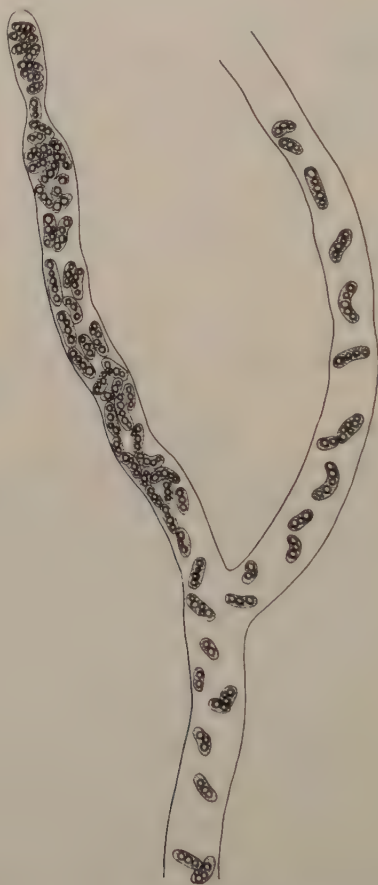


Fig. 1. Drawing of a portion of a living colony of *Thiodendron mucosum* to show distribution of sulphur and cells within the mucus. $\times 1600$.

The organism, which will be referred to as *Thiodendron*, consists of rod-shaped bacteria $3-9\mu$ long and $1.0-2.5\mu$ in diameter, forming dendroid colonies up to 3000μ across, in a jelly-like matrix (Pl. 1, figs. 1, 2, 4; Fig. 3). In this jelly, the individual organisms are distributed at random, but tend to be most numerous near the ends of the colony. Most of the organisms are of similar size ($3-5\mu$ long $\times 1.5\mu$ diameter), but often the organisms at the swollen ends of the branches are three times this size (Fig. 3). These large forms appear to be typical of older colonies, because quite young colonies, although they may have swollen tips, normally

exhibit only smaller forms. The smaller forms usually have 3 to 8 sulphur granules which vary in size, but which are generally arranged along the linear axis. The organisms in the larger tips may have as many as 20 sulphur granules which vary in size, but which are generally arranged along the linear axis, rather crowded, and when a tip is crowded with organisms it is much darker than the distal part of the colony (Fig. 3).

Evidence that the granules are indeed sulphur is based upon the type of the inclusion, round bodies with uniform black edges (the 'droplets' of Winogradsky), and upon the recovery of sulphur on evaporating extracts of the colonies with carbon disulphide. The colony does not show branching until it is at least several cell-lengths long. While there is no definite pattern for the arrangement of the bacteria within the common jelly, most of the bacteria have their long axis parallel to the filament axis.

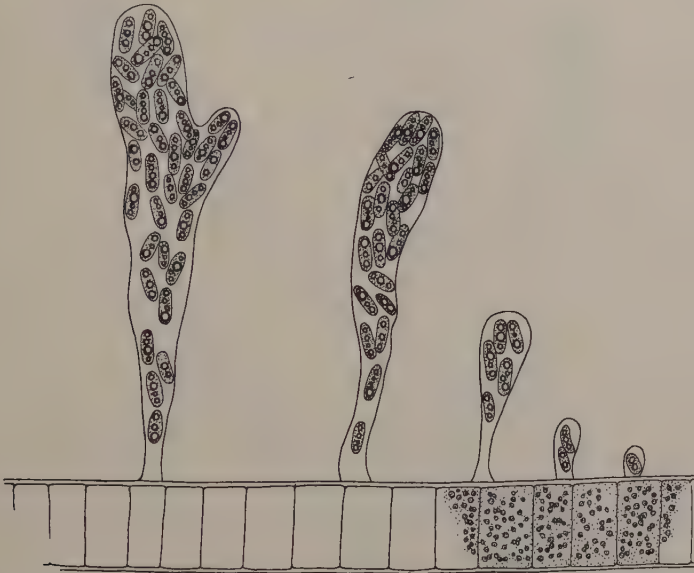


Fig. 2. Drawing of five young living colonies of *Thiodendron mucosum* attached to a filament of *Lyngbya*. $\times 1600$.

Outlines of the bacterial forms are very difficult to see. In fact, their shape was first suggested by the linear clumps of sulphur granules (Fig. 1). Their internal make-up is quite homogeneous, with very nearly the same optical density as the jelly. One gets the impression of branching jelly crowded with sulphur granules. However, the bases of the mucous fingers are frequently almost devoid of bacteria. Also, when colonies are left under a cover glass in a moist chamber, after 24 hr. they begin to lose their sulphur, and pale ghosts of the bacteria become visible. Sometimes bacterial forms showing a constriction may be seen, as if they were undergoing binary fission. The bacterial forms stain very poorly, if at all, by Gram's method. Acids cause swelling and disintegration of the mucilage, and distort it. The bacterial forms are not coloured by iodine.

Older colonies are quite arboroid in habit (Pl. 1, figs. 1, 2, 4). Superficially they resemble *Zooglea ramigera*. A given colony tends toward uniformity in the size of its club-shaped parts (Fig. 3). Colonies over 2 mm. across have been found on many occasions. No holdfasts have ever been detected, but the attachment is strong. A colony brushed by a rotifer or ciliate bends, but is not detached. Usually attachment is to a filament of *Lyngbya* (Fig. 2), but occasionally it is to some other surface. Plate 1, fig. 2, shows a colony attached to the stalk of a colonial vorticellid. Colonies



Fig. 3. Portion of a mature living colony of *Thiodendron mucosum* to show the larger bacterial forms near the swollen ends of branches. $\times 1600$.

evidently start from a single motile organism which settles down and grows out. Colonies with as few as three bacterial forms (Fig. 2) may be seen, already becoming club-shaped. Bacterial forms leaving the jelly of a colony have not been seen.

The organism has not been successfully cultivated in the laboratory. Colonies remain intact, but with no growth for several days, in a moist chamber. During this time, the sulphur granules gradually disappear. The temperature of Warm Mineral Spring is a uniform 84°F. (29°C.), and that of the intracoastal waterway at a time when the organism was taken there was 55°F. (13°C.).

Colonies have been kept apparently thriving for 3 months at 72°F. (20°C.) in

jars of Warm Mineral Spring water, but colonies picked out and transferred to various liquid culture media invariably died within a few days. No increase in visible sulphur content, nor in viability resulted when such colonies were exposed to 0.3–3.0 % of hydrogen sulphide. This species did not show an ability to oxidize hydrogen sulphide under the conditions in the laboratory, being different from *Sphaerotilus* in this respect, as reported by Skerman, Dementjeva & Carey (1957), and by Waitz & Lackey (1959). Scotten (1951) also reported that *Beggiatoa* did well when small amounts of hydrogen sulphide were present. More recently Faust & Wolfe (1961) secured pure cultures of *Beggiatoa* without adding hydrogen sulphide, and we normally obtain massive cultures of *Beggiatoa* with other organisms in the same manner. However, every time the organism *Thiodendron* has been taken, enough H_2S was present in its environment to be detectable by its odour. Hydrogen sulphide is present constantly in the Warm Mineral Spring. Sulphide determinations show amounts varying around 0.1 p.p.m.; calculations show H_2S at about 0.16 p.p.m., and HS about 0.08 p.p.m. These are manifestly considerably below limiting factors for many organisms, certainly for *Thiodendron*.

The organism *Thiodendron* is another addition to the long list of parallel forms found in the lower plants. The organism essentially is a collection of rod-shaped bacterial forms in a common jelly, apparently not organically connected to each other. The same is true of *Zooglea ramigera*, whose cells are smaller and without sulphur. Even the dendroid habits of the two organisms are similar. Except for the branching of the jelly, almost the same comparison could be made with the blue-green algae *Aphanotheca* and *Bacillosiphon*. Pringsheim (1949) discussed possible relationships between bacteria and *Myxophyceae*, and this new organism adds material for that discussion.

Taxonomic position of Thiodendron

The organism occurs in sufficient abundance and is sufficiently distinctive to represent a valid species. Its resemblance to *Zooglea* is superficial, and the latter never accumulates sulphur, although a number of experiments has been made in attempts to induce this. The new organism does not accumulate iron in its sheath and does not resemble other jelly-inhabiting bacteria such as *Nevskia* or *Leucocystococcus*. Devidé (1952) described as new colourless sulphur-accumulating bacteria, *Thioglea*, which live within jelly, but these are non-branching, oval or elongate masses. They would seem to be the closest relatives of *Thiodendron* even to the detail that cell outlines are difficult to make out. However, Devidé thinks they might belong to the *Achromatiaceae*, which *Thiodendron* obviously does not.

The accumulation of sulphur granules within the cell is the most striking characteristic of *Thiodendron*. This is true of *Beggiatoa*, *Thiothrix*, *Thioploca* and *Thiospirillopsis*. *Thiothrix* is attached; the others move about freely. The cells of each of these (if colonial; *Thiospirillopsis* is not) are in organic apposition, whereas *Thiodendron* cells may be far removed from each other, in older colonies. The family *Beggiatoaceae*, then, shows considerable variation among its respective genera, and this new genus is certainly a related one. In *Achromatium* there are free sulphur granules, but also large calcium carbonate inclusions and the cells are without jelly and freely motile. It does not seem that *Thiodendron* is closely related to this genus. Neither does it seem related to the families *Vitreoscillaceae* and *Leucotrichaceae*.

The only other possible relative seems to be *Thiobacterium bovista* of the family Thiobacteriaceae. This species is rod-shaped, about the same size and is described as having one to four sulphur granules within each cell. Furthermore it is colonial, but the colonies are bladder-like. Thiodendron in some respects is quite similar. But it has few similarities to Thiospira or Thiobacillus, the other members of the family Thiobacteriaceae.

Thiodendron never shows colour and cannot be mistaken for a blue-green alga or any of the Thiorhodaceae. It is therefore considered a new genus and species for which the name *Thiodendron mucosum* is proposed. This is from the Greek nouns thion, sulphur, and dendron, tree (a sulphur tree) and the Latin adjective mucosus, slimy. Thiodendron is neuter. Pringsheim, when he saw the organism, suggested Thiobrachys for it, but this proved to be etymologically unacceptable. The taxonomic position of many of the sulphur bacteria seems a matter of diverse opinion, as shown by Bisset & Grace (1954). This one seems completely unrelated morphologically to many Beggiatoales and to the Achromatiaceae; in addition, no movement of the bacterial forms within the jelly, or of the colony as a whole, has been observed. Its lack of colour is enough to exclude it from the Thiorhodaceae or the Athiorhodaceae, even though its sulphur relationships are still not understood. It seems preferable to place it in the order Beggiatoales despite its apparent lack of motility in vegetative cells. However, a new family, Thiodendraceae, is proposed and it is hoped that the organism may become better known.

Habitat and associations

The occurrence of a unique organism in a unique environment usually brings the question 'What factors are responsible for its presence?' It was at first thought that the half sea-water concentration, H_2S without pollution, and the temperature in Warm Mineral Spring were responsible for the occurrence of Thiodendron, but then it was found at Titusville. No analysis of the water there is available, but its salinity was found to be about 17,000 p.p.m. The water temperature at the time of collection was 55° F. (12.5° C.) and there were ample visual evidences of organic pollution. In June 1959 Pagosa Hot Spring in Colorado was examined; this spring is hot to warm, and has a high salt content. *Beggiatoa* and *Thiothrix* species occurred there, but no Thiodendron in the samples taken. At present physicochemical factors do not explain its occurrence.

However, Warm Mineral Spring has a unique flora and fauna; it seems ideal for sulphur bacteria. All species of *Beggiatoa* are abundant; *B. gigantea*, which has not been recorded from fresh water and which we have not found elsewhere in dense aggregates, may be scooped up there in great handfuls. *Symploca* has not been found, but many other groups are (see Table 3). *Thiospirillopsis floridanus*, described by Uphof (1927) from Welaka, Florida, 200 miles north, has been found once in some thirty trips to Warm Mineral Spring. It is thus recorded now (the second record?) from brackish as well as fresh water.

In Warm Mineral Springs the blue-green algae are unusual, so are the diatoms, green algae and flagellates; few filamentous green algae occur and only an occasional green euglenid has been taken. The dominant green flagellate is *Trentonia flagellata*, which apparently had not been seen until the present work since it was described by Stokes (1886); Hollande (1952) called it a synonym of *Vacuolaria*, but the original

description is valid. Incidentally, it was also a dominant near the bottom of Green Hill Pond, a Rhode Island salt pond of low salinity, in July 1960. The rhizopods are few, but include two Foraminifera. The ciliata are mostly salt-water types, and include several undescribed species. Little work has been done with other organisms, but some flatworms are new and a new nereid was described by Hartman (1959), while two of the three microcrustacea are species which Dr H. C. Yeatman (personal communication, 1959) believes to be distinct undescribed species. Many other groups are represented by few species, or are absent. The whole picture is of a very unusual environment, which has produced unusual organisms.

Table 3. *The number of genera or species of various organism groups found at Warm Mineral Spring*

About 5 % have not been identified and there are some which are new. All identifications were made upon living organisms by direct microscopic examination

Sulphur bacteria	34	Dinoflagellata	6
Blue-green algae	27	Bacillarieae (Diatoms)	20
Green algae and Charales	6	Zooflagellata	24
Euglenida	24	Rhizopoda	26
Chloromonadida	1	Ciliata	99
Cryptomonadida	3	Metazoa	32

DISCUSSION

It seems reasonable to suppose that an organism is a product of its environment; on this score a highly specialized environment such as Warm Mineral Spring might reasonably be supposed to have the very special or rare organisms such as *Thiodendron* appears to be. Factors common to Titusville and Warm Mineral Spring appear to include degree of salinity, presence of H_2S and some few related characteristics. But the environment at Titusville is not constant, so there the comparison ends. *Thiodendron* is such a striking organism that were it not greatly restricted in occurrence it would have been reported long before this.

In this case, the particular organisms present in abundance, and constantly, emphasize the importance of sulphur, especially hydrogen sulphide, in the energy relationships of a given locale. These energy relationships may be expressed by the formula: $H_2S \rightarrow S + 2H + 2e$. The free energy liberated by this reaction is -7.89 Cal. Lamanna & Malette (1953) state that the Beggiatoaceae use as an energy source the oxidation of H_2S . Since H_2S oxidizes readily in the presence of minute quantities of metals, Warm Minerals Spring with a flow of about 9,000,000 gallons daily, and an H_2S content of 0.162 mg./l. provides a large source of energy to those micro-organisms able to obtain it. Some of the organisms found in Warm Mineral Spring are ubiquitous; others are characteristic of an environment containing H_2S . When a jar containing a large variety of Warm Mineral Spring organisms is tightly closed, most of the organisms die within 24 hr. Table 4 gives a list of the resistant organisms.

There are many organisms which use soluble sulphur directly in their metabolism. But there are also many which ingest either the free sulphur granules, or sulphur bacteria. With some ciliates, ingested bacteria may be clearly observed in food vacuoles. *Frontonia* ingests some of the smaller Beggiatoales; others ingest *Thiovulum*, *Chromatium* or *Macromonas bipunctata*. This ingestion suggests a mechanism for the transfer of S from H_2S to the soil as elementary sulphur. Beggiatoa (and

similar bacteria, perhaps including *Thiodendron*) oxidize H_2S to S: this may be oxidized in turn to H_2SO_4 , but in an alkaline medium (the sea, Warm Mineral Spring) the acid is neutralized. When the bacteria are ingested, there may be a direct deposition of their contained elementary S in the soil. Indeed, free S is readily found in bottom deposits at Warm Mineral Spring. The water there contains adequate carbonates, and at least 0.05 p.p.m. NO_3 , also CO_2 , so that respiration is no problem, yet dissolved oxygen remains low. There is a high organic content in Warm Mineral Spring water which evidently serves as food for the micro-organisms. Considerable quantities of *Chara* and four genera of blue-green algae, *Chroococcus*, *Gomphosphaeria*,

Table 4. *Organisms alive after 48 hr. saturation of Warm Mineral Spring water with hydrogen sulphide*

All identifications based upon direct microscopic examination.

1. <i>Beggiatoa alba</i>	17. <i>Navicula</i> spp.
2. <i>B. arachnoidea</i>	18. <i>Petalomonas</i> sp.
3. <i>B. gigantea</i>	19. <i>Gymnodinium</i> sp.
4. <i>B. leptomitiformis</i>	20. <i>Cyathomonas truncata</i>
5. <i>B. minima</i>	21. <i>Bicoeca lacustris</i>
6. <i>B. mirabilis</i>	22. <i>Bodo reniformis</i>
7. <i>Chromatium</i> spp.	23. <i>Monas vulgaris</i>
8. <i>Chroococcus turgidus</i>	24. <i>Spirochaeta</i> spp.
9. <i>Gomphosphaeria aponina</i>	25. <i>Amoeba</i> sp.
10. <i>Lyngbya</i> sp.	26. <i>Cryptopharynx</i> sp.
11. <i>Oscillatoria</i> spp.	27. <i>Cyclidium</i> spp.
12. <i>Spirulina major</i>	28. <i>Epalxis exigua</i>
13. <i>S. princeps</i>	29. <i>Metopus es</i>
14. <i>Amphiprora</i> sp.	30. <i>M. intercedens</i>
15. <i>Amphora ovalis</i>	31. <i>M. vestitus</i>
16. <i>Biddulphia</i> sp.	32. <i>Parablepharisma peltitum</i>

Aphanocapsa and *Oscillatoria* grow in the shallow areas, but can hardly act as primary producers. Instead, the sulphur bacteria whose biomass is much greater probably use soluble organic matter of unknown nature and become primary producers. We have recently found that ^{14}C is taken up from urea and glucose present in a medium containing living *Thiothrix*. The low dissolved oxygen in Warm Mineral Spring and its run is easily compensated for by the presence of oxidized sulphur and oxygen-containing organic compounds as long as there is biological action which is easily demonstrated.

This work was done under a grant-in-aid from the National Institutes of Health, U.S. Public Health Service.

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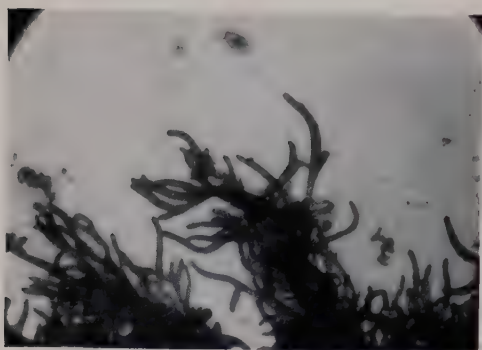


Fig. 1

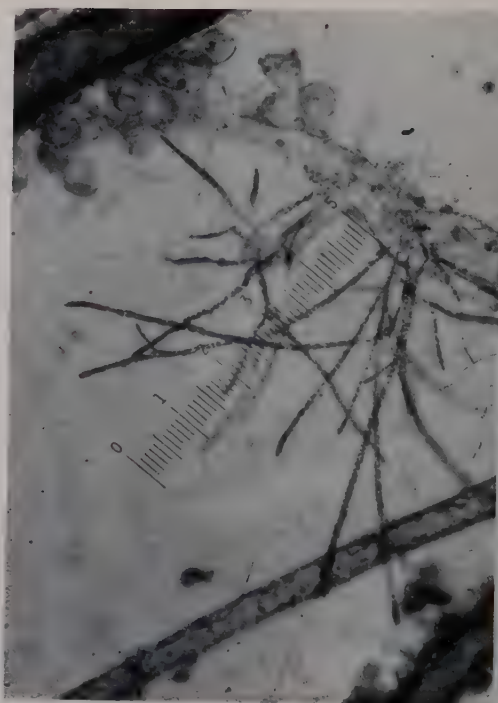


Fig. 2

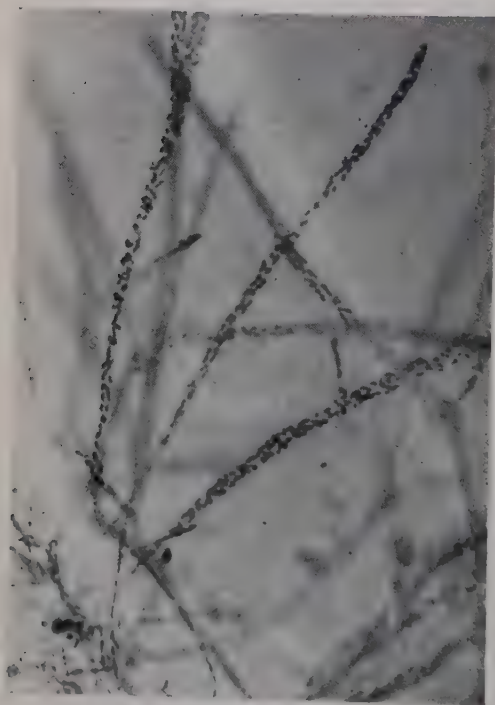


Fig. 3

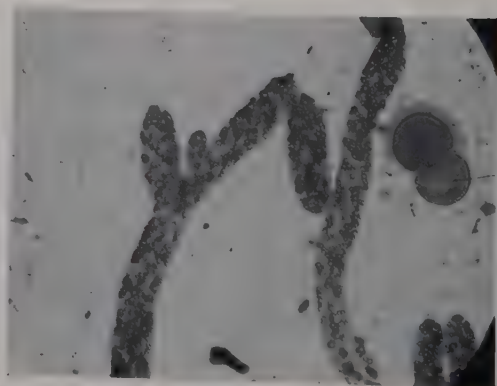


Fig. 4

J. B. LACKEY AND E. W. LACKEY

(Facing p. 39)

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EXPLANATION OF PLATE

- Fig. 1. Portion of a mature colony of *Thiodendron mucosum* to show branching. Colony stained while living with aqueous safranin. $\times 400$. Photographed with Kodak 682 C, $\frac{1}{250}$ sec., at f. 4.5.
- Fig. 2. Photograph of an old colony of *Thiodendron mucosum* attached to the stalk of a colonial vorticellid. $\times 400$. Photographed with Zeiss Contaflex IV using Dupont SX-Pan film, ASA-4000, $\frac{1}{250}$ sec. at f. 2.8.
- Fig. 3. Portion of a mature colony of *Thiodendron mucosum* to show the distribution of sulphur in the colony fingers. Note the frequent linear aggregates, which indicate the long axes of unstained individual cells. The black pointer at the left ends on a *Chroococcus turgidus* colony (Blue green alga). $\times 750$. Photographed with Kodak 682 C, $\frac{1}{250}$ sec., at 4.5.
- Fig. 4. Photograph of a mature living colony of *Thiodendron mucosum*, to show its arboroid appearance. $\times 400$. Photographed with Speed Graphic, Century, $2\frac{1}{4} \times 3\frac{1}{2}$ using Ansco Super Hypan film, $\frac{1}{250}$ sec., at f. 4.5.

Torulopsis castellii sp.nov., a Yeast Isolated from a Finnish Soil

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(Received 10 January 1961)

SUMMARY

A new species of *Torulopsis* is described; it was isolated from a Finnish soil. This species is named *T. castellii* in honour of Professor Tommaso Castelli of the Agricultural University of Perugia, Italy.

INTRODUCTION

In a microbiological study of 32 samples of Finnish soil taken in August 1957, 22 species of yeasts were isolated; 15 of them were referred to an already described species, while 7 had to be considered as new species or varieties. The present paper describes a new species belonging to the genus *Torulopsis* for which the author proposes the name *Torulopsis castellii* in honour of Professor Tommaso Castelli, Director of the Istituto di Microbiologia Agraria e Tecnica of Perugia University, Italy.

METHODS

Origin of strains. Seven strains were studied, all isolated from a soil sample from Hyytiala in the Province of Tavastia Australis, Finland. The soil was from a pine bog with underbrush taken at a depth of 2-10 cm. It was tan coloured, humiferous, without CaCO_3 and at pH 3.7. The methods used to study the characters of the yeasts were those of Lodder & Kreger-van Rij (1952). In examining the utilization of sugars, the boiled and washed agar method described by Capriotti (1955) was used.

RESULTS

Growth in grape must. After 3 days at 25°, the cells oval; $1.8-3.5 \times 2.2-4 \mu$; single or in pairs or in chains of 3-10 cells. After 1 month at 17°: a thin ring, more or less limpid liquid, and sediment well developed.

Growth in malt extract. After 3 days at 25°, the cells oval; $2-3.5 \times 2.3-4.3 \mu$; single or in pairs or in chains of 7-16 cells. After 1 month at 17°: a trace of ring, limpid liquid, and sediment well developed (Fig. 1).

Growth on malt agar. After 3 days at 25°, the cells oval, elliptical to long narrow $1-3.5 \times 2-5 \mu$, single or in pairs. Streak culture white gray, glistening, smooth, flat, waxy, rather thin, with smooth margin. After one month at 17°, streak culture white-yellowish, glistening, smooth, rather flat, not well developed, with smooth margin.

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Grape must + gelatine stab. After 60 days, at 18–20°, superficial growth only of colony, white-ochraceous, expanded, flat; gas bubbles formed; no liquefaction.

Sporulation. Ascospores not formed.

Giant colony on grape must + gelatine. After 1 month at 18°, the giant colony appeared round with large and smooth cavity, and slightly lobate crown.

Pseudomycelium. In slide culture, pseudomycelium not formed.

Fermentation. Glucose well fermented; galactose, maltose, sucrose, lactose, trehalose, raffinose, inulin, dextrin not fermented.

Assimilation of carbon compounds. Glucose, trehalose (slowly), D-ribose (very slowly), glycerol well assimilated. Not assimilated: galactose, maltose, sucrose, raffinose, lactose, L-sorbose, cellobiose, melibiose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, L-rhamnose, D-glucosamine HCl, i-erythritol, adonitol, mannitol, D-sorbitol, α -methylglucoside, salicin, potassium-D-gluconate, potassium-5-keto-D-gluconate, potassium sodium-saccharate, pyruvic acid, D-lactic acid, succinic acid, citric acid, ethyl acetoacetate, i-inositol.

Assimilation of nitrogen compounds. Ammonium sulphate assimilated; potassium nitrate not assimilated.

Splitting of arbutin. Negative.

Fermentative power in grape must. Ethanol produced 5.3–7.8% (v/v).

DISCUSSION

The isolated strains are referred to the genus *Torulopsis* because the cells are oval and form no spores, pseudomycelium or carotinoid pigments. Lodder & Kreger-van Rij (1952) reported in the genus *Torulopsis* only one species with good fermentation and assimilation of glucose, namely, *T. glabrata* (Anderson) Lodder & De Vries, which was originally isolated by Anderson from human faeces, with the name of *Cryptococcus glabratus*. However, this species also fermented trehalose. More recently other species have been described which ferment only glucose; but these species are different from *T. castellii* in the following characters: *T. pintolopesii* Van Uden (1952) assimilates only glucose, does not assimilate glycerol, D-ribose or trehalose; does not grow at 24°; *T. nitratoiphila* Shifrine & Phaff (1956) ferments glucose (latent); assimilates glucose, galactose, sorbose, trehalose, xylose, L-arabinose, D-arabinose, mannitol, sorbitol, adonitol, glycerol; does not split arbutin; *T. wickerhamii* Capriotti (1958) ferments glucose well; assimilates glucose, galactose, potassium nitrate; splits arbutin.

Two strains of *Torulopsis castellii* sp. nov. have been sent to the Centraalbureau voor Schimmelcultures, Delft, Netherlands; 7 strains have been put in the yeast collection of Istituto di Microbiologia Agraria e Tecnica dell'Università di Perugia, Italy.

TORULOPSIS CASTELLII SP. NOV.

Maltato in musto cellulae ovaes 2–3.5 × 2.3–4.3 μ , singulae, binae. Ruente mense (17°) sedimen, medium nitidum et annulum.

Maltato in agaro trium dierum (25°) cellulae ovatae, ellipticae etiam angustae 1–3.5 × 2–5 μ , singulae aut binae. Elapso mense fit patina albaflava, cerea, lucida, laevigata, non copiosa. Nulla sporificatio observatur. Pseudomycelium deest.

Glucosum fermentatur. Assimilantur glucosum, glycerina, trehalosus (lente) et D-ribosum (lente). Nitras kalicus non assimilatur.

Minerali in medio cum alcohole aetilico ut unum carbonii pabulum, non augescit. Alcohole aetilicus gignit 5.3–7.8 %. Arbutinum non finditur. E terra Finnica se-junta fuit. Typus: cultura 211 iacens in C.B.S., Delft, Holland.

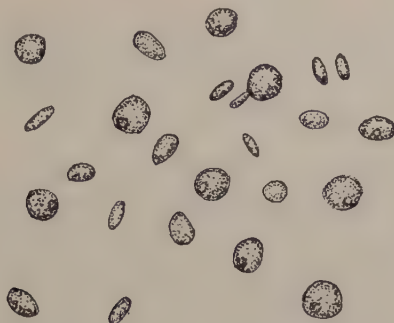


Fig. 1. *Torulopsis castellii* nov.spec. ($\times 700$) (after 3 days on malt extract).

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A Growth Medium without Blood Cells for *Pasteurella tularensis*

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SUMMARY

A modification of glucose cysteine blood agar (GCBA) is described for the cultivation of *Pasteurella tularensis* in which the blood is replaced by plasma and catalase. It has the advantage for colony counting of being clear, and is at least as good as GCBA for enumerating fastidious aged *P. tularensis* suspensions and aerosols. The liquid medium supports the growth of very small inocula.

INTRODUCTION

Glucose cysteine blood agar (GCBA) was described by Downs, Coriell, Chapman & Klauber (1947) as a suitable medium for the enumeration of *Pasteurella tularensis* by a plate colony counting method. The medium gives satisfactory counts in experienced hands but the need to use blood presents two problems: control of its quality is difficult, and it renders the medium opaque and therefore less satisfactory for colony counting. Won (1958) described a blood-free medium, but we have found it unable to promote full growth of aged organisms from stored suspensions or aerosols. This paper describes a clear medium which promotes the growth of *P. tularensis* as well as GCBA and gives consistent results.

METHODS

Glucose cysteine blood agar

GCBA was used throughout the work as the control medium against which variants of it were tested. The formulation was changed somewhat from that of Downs *et al.* (1947). The basal medium contained (g./100 ml.): Lab-Lemco (Oxoid), 0.3; peptone (Evans), 0.5-3.0; sodium chloride (Analar), 0.5; agar (Davis), 1.25; distilled water to 100 ml. The medium was completed by the addition of separately autoclaved solutions of L-cysteine HCl (10%) 1 ml.; L-histidine HCl (10%) 1 ml.; glucose (50%) 5 ml.; and 4 ml. of whole human blood (citrated).

The optimum amount of peptone was found to vary from batch to batch. The histidine decreased the required incubation time by several hours.

The basal medium was prepared by dissolving the Lab-Lemco, peptone and salt and adjusting to pH 7.6-8.4 (according to peptone batch and concentration) with sodium hydroxide so that the medium when completed was at pH 6.8-7.0. The agar was then added and, after steaming to melt it, the basal medium was autoclaved in convenient amounts for 15 min. at 120°. No deterioration of the basal medium was found to occur for at least 1 year when stored in screw-capped bottles in the dark at room temperature.

The medium was completed by melting the basal portion, cooling to 50–55°, and adding the remaining ingredients glucose, cysteine, histidine and finally blood. Penicillin to 10 units/ml. was frequently added; this was desirable to inhibit contaminants in certain uses of the medium, and had no detectable effect on the growth of *Pasteurella tularensis*. Petri plates were poured and 'dried' in the usual way for about 2 hr. at 37°.

Organism. Small-scale preparations were made on a laboratory shaker, using a modified casein partial hydrolysate medium. Strain Schu D was used for most of the work, and four other strains in confirmatory tests. The suspensions were stored at 0 to +4°.

Viable counts. Viable counts were made by the drop technique of Miles & Misra (1938). Dilutions were chosen to give about 150 colonies/plate on the control medium; six plates of each medium under test were counted. Colonies were counted when they reached 1–2 mm. diam. which was usually after 2 days at 37°; with some media longer incubation was necessary.

Use of 'aged' organisms. In the early stages of the work it was found that suspensions which had been stored longer than 3 or 4 weeks, and also samples from some 'aged' aerosols, were much more exacting in their requirements for growth than were 'young' suspensions or aerosols of high viability. Media were therefore tested with the more fastidious aged suspensions and sometimes also with aerosol samples. This was important, since media which were satisfactory in all other respects sometimes failed in this test.

RESULTS

Plate counts on the control GCBA medium were scaled to 100, and counts on test media in the range 85–115 were not considered significantly different from the control. A few typical results are shown in Table 1.

During preliminary work it was found that growth was completely inhibited when the glucose was autoclaved in the basal medium; this inhibition was partly annulled when the cysteine also was autoclaved in the basal medium. No inhibition was found when cysteine alone was autoclaved in the basal medium. Nevertheless, because of its instability it was decided to add it separately to the medium when required. A 10% solution of L-cysteine autoclaved 15 min. at 120° in a screw-capped bottle lost 8% of its cysteine. This loss could be safely ignored since it was found that the cysteine at 1000 µg./ml. in the medium was about 100 times minimal requirement: suspensions of *Pasteurella tularensis* up to 33 weeks old and aerosol samples of less than 0.5% viability gave plate counts equal to the control with cysteine down to 10 µg./ml. Cysteine was found to promote better growth than cystine in plain (i.e. no blood) agar, confirming the work of Downs *et al.* (1947). No difference could be demonstrated between them, however, when used at concentrations 1–1000 µg./ml. in the medium with blood. It was apparent that not only did blood eliminate the difference between growth promotion of the amino acids but it also stimulated the growth of aged *P. tularensis* which failed to grow on plain cysteine or cystine agar.

Attention was then turned to the blood. We usually used citrated whole human blood. Neither the cells nor the plasma would alone support full growth of aged *P. tularensis*.

Lysis of erythrocytes takes place in a relatively short time even in cold storage,

and it was found that lysed blood was inferior to non-lysed blood when tested with aged *Pasteurella tularensis*. Erythrocytes were lysed in distilled water and washed by centrifugation until there were no intact cells in the deposit and the supernatant fluid was colourless. The deposit was used in the same proportion as the stroma content of whole blood in the control medium, and with 4% (v/v) human plasma it gave growth equal to the control; with 2% (v/v), the count was about 75% of control. The evidence suggests the presence of inhibitors in the erythrocytes rather than loss of growth factors when the erythrocytes were lysed.

Table 1. *Growth of Pasteurella tularensis on glucose cysteine agar with various additions*

Addition	Age of suspension (weeks)		
	6	15	20
	% growth		
None	0	0	0
4% human blood (control)	(100)	(100)	(100)
Human blood cell stroma (from 4% human blood)	101	55	25
Human blood cell stroma and 2% human plasma	92	74	74
Human blood cell stroma and 4% human plasma	102	90	90
4% human plasma and 10 μ g./ml. catalase (crude)*	101	68	45
4% human plasma and 100 μ g./ml. catalase	107	75	67
4% human plasma and 300 μ g./ml. catalase	90	107	106
4% horse plasma and 300 μ g./ml. catalase	105	—	97
300 μ g./ml. catalase + 4% reconstituted freeze-dried human plasma	98	93	95
300 μ g./ml. catalase + 4% dialysed human plasma	93	101	94

* About 1 *Kat.f./mg.*

The growth-promoting action of the stroma seemed likely to be enzymic, and as it was found to have retained some catalase action preparations of this enzyme were tested. An impure beef-liver catalase extract of activity about 1 *Kat.f./mg.* was Seitz-filtered in 1% (w/v) solution in phosphate buffer (pH 7.4) and added to the medium, together with human plasma, in place of whole blood. With this catalase preparation at 300 μ g./ml. + 4% (v/v) plasma, the *Pasteurella tularensis* counts were equal to that of the control. The activity of the catalase required was about equal to that of 4% (v/v) of whole blood and much more than that of the separated stroma. The function of the stroma therefore cannot be attributed solely to the catalase content. Catalase was, however, capable of completely replacing the stroma. It was shown by tests with pure catalase that it was the enzymic activity that was responsible. Pure crystalline catalase (about 100 *Kat.f./mg.*) was equal in effect, at 3 μ g./ml., to the crude catalase (about 1 *Kat.f./mg.*) at 300 μ g./ml.

In the first tests with the 'blood-free' medium, citrated human plasma (about 4%, v/v) was used + 300 μ g./ml. crude catalase preparation. This plasma could be replaced by reconstituted freeze-dried human plasma and by human plasma dialysed against physiological saline. Ox plasma or horse plasma were also suitable but the pH value was rather critical at about pH 6.7, in contrast to human plasma which was satisfactory within the range pH 6.5–7.0.

Recommended medium

The composition of the medium finally adopted was as follows (g./100 ml.): Lab-Lemco (Oxoid), 0.3; peptone (Evans), 0.5-3; sodium chloride, 0.5; agar (Davis), 1.25; distilled water to 100 ml. The following ingredients were added separately as sterile solutions: glucose, 5 ml. (50 % solution); L-cysteine HCl, 1 ml. (10 % solution); L-histidine HCl, 1 ml. (10 % solution); catalase (pure), 0.1 ml. (1 % solution); plasma (human, ox, horse), 5 ml., pH 6.6-6.8. The method of preparation for this medium was as for GCBA. It should be noted that the catalase content given is about three times the adequate concentration of $3\mu\text{g.}/\text{ml.}$; this margin of safety may not be necessary. Poured plates of this medium keep about as well as GCBA when stored at 4°. For routine use plates kept for 2 weeks were satisfactory, but for extremely sensitive aged organisms 1 week was the limit.

The medium was tested with *Pasteurella tularensis* aerosols which had been held until the viability was considerably decreased (as determined on GCBA by the method of Harper, Hood & Morton, 1958). The bacteria remaining viable in such aerosols are in a sensitive state, and make demands on the culture medium that are similarly exacting to those of aged suspensions. In a series of five experiments, 12,376 colonies were counted on the new medium and 11,215 on the control, the ratio of 1.1 showing a small (possibly not significant) advantage in favour of the medium described here. The new medium was tested with aged suspensions of four other strains of *P. tularensis* (Fam, Jap, 403, and LV), and the results confirmed the performance with Schu D.

The success of the solid medium in growing *Pasteurella tularensis* suggested trial of the liquid medium, without agar, in shaken cultures. The yields of organisms obtained were similar to those from the casein hydrolysate medium (about 4×10^{10} organisms/ml.). Unlike the casein hydrolysate medium the new medium supported growth from small inocula of less than 10 organisms/ml.

My thanks are due to Mr I. H. Silver for helpful criticism, Mr L. Flower for technical assistance, and Dr H. T. Eigelsbach (Fort Detrick, Frederick, Maryland, U.S.A.) for supplying strains of *Pasteurella tularensis*.

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Purification of Factor I and Recognition of a Third Factor of the Anthrax Toxin

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With a note by H. SMITH, K. SARGEANT and J. L. STANLEY about serological precipitation in gels as a criterion of purity of antigens

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SUMMARY

Factor I of the anthrax toxin was isolated and showed one major component in the ultracentrifuge and on paper electrophoresis; it contained less than 5.5% of extraneous antigens detectable by serological precipitation in gels. The final preparation contained all the usual amino acids (N = 10.1%) and some carbohydrate (6%, calculated as glucose) and phosphorus (0.7%). The most striking aspects of its analysis were a high ash (10-13%) and a light absorption at 260 m μ . The high ash was not due to one element but to a highly variable metal content (mainly Ca, Mg, Ni, Cu) indicating a powerful and indiscriminate chelating action of factor I. This chelating action might have been due to the chemical entity which absorbed light at 260 m μ and which was not RNA or DNA.

The final preparation of factor I was not toxic when injected alone but when mixed with purified factor II it evoked oedema in the skin of a rabbit and killed mice. However, the concentration of this mixture which killed mice formed a much larger skin reaction in rabbits than a comparable dose (based on mouse LD₅₀) of either crude toxin or a mixture of crude factors I and II. An investigation of this fact led to the demonstration and partial purification of a third factor (III) of the anthrax toxin which: (1) was different serologically from factors I and II; (2) was present in anthrax toxin produced *in vivo*; (3) was non-toxic when injected alone; (4) was lethal for mice when mixed with factor II but not with factor I; (5) increased the lethality of mixtures of factors I and II for mice and decreased their capacity to produce oedema in the skin of rabbits. A mixture of factors I, II and III showed synergic action in toxicity tests in mice; the mixture killed guinea pigs which showed signs of oligæmic secondary shock (as did guinea pigs killed by anthrax infection).

INTRODUCTION

The specific lethal and oedema-forming toxin of *Bacillus anthracis* was first recognized *in vivo* and then produced *in vitro*; it consisted of at least two components—factors I and II—which acted synergistically (Smith, Keppie & Stanley, 1955*a*; Smith *et al.* 1956; Harris-Smith, Smith & Keppie, 1958; Thorne, Molnar & Strange, 1960; Stanley, Sargeant & Smith, 1960; Sargeant, Stanley & Smith, 1960). This paper describes the purification, biological action and chemical properties of factor I of anthrax toxin.

A mixture of the final preparation of factor I + purified factor II resembled crude toxin in producing oedema in the skin of a rabbit and in killing mice, but its behaviour was not identical with that of the original toxin. The concentration of the mixture of purified preparations which killed mice formed a much larger skin reaction in rabbits than a comparable dose (based on mouse LD₅₀) of either crude toxin or a mixture of crude factors I and II. An investigation of this result led to the recognition of a third factor of the anthrax toxin which is described in this paper.

METHODS

Assay for factor I of the anthrax toxin. This was as described by Stanley *et al.* (1960).

Tests for immunogenicity. These were carried out in guinea pigs and rabbits as described by Smith & Gallop (1956) and Strange & Thorne (1958).

Tests for lethality to mice and guinea pigs. These were as described by Smith *et al.* (1955a).

Tests for oedema production in rabbit skin. These were as described by Smith *et al.* (1955a) for guinea pigs, including the nomenclature used to describe the size of the skin lesion.

Serological precipitation in gels. Unless stated otherwise the anthrax antisera ('spore' H. 533; 'antigen' H25) and methods used were as described by Sargeant *et al.* (1960).

Diethylaminoethyl cellulose (DEAE-C, Peterson & Sober, 1956). This was bought from Eastman Kodak Ltd.

Analytical ultracentrifugation. We are indebted to our colleagues Dr K. Cammack and Mr K. Grinstead for these observations in the Spinco Ultracentrifuge Model E.

Paper electrophoresis. Samples were electrophoresed at two pH values (0.2 μ barbitone buffer pH 8.6; 0.2 μ acetate buffer pH 5) on Whatman no. 3 paper for 18 hr. with a potential gradient of 10 V./cm. and in a cooled apparatus; the papers were stained with naphthalene black.

Nitrogen. This was determined by the Kjeldahl (Kj.) and Dumas (D) methods.

Total carbohydrate. This was estimated by the orcinol method of Sørensen & Haugaard (1933).

Phosphorus. This was measured by the method of Fiske & SubbaRow (1925).

Lipid. Samples were hydrolysed with N-HCl for 1 hr. at 100° and evaporated to dryness at room temperature over P₂O₅ and NaOH. The residue was extracted by the method of Folch, Lees & Sloane Stanley (1954); the material soluble in chloroform was weighed.

Hexosamine. This was determined by the Elson & Morgan method.

Ash. This was sulphated.

Protein. This was estimated for chromatographic purposes by the method of Lowry, Rosebrough, Farr & Randall (1951) with ovalbumin as a standard.

Amino acids. A hydrolysate (16 hr., 100°, 6N-HCl) was examined for amino acids by two-dimensional paper chromatography, with phenol and collidine as solvents.

Absorption of ultraviolet (u.v.) radiation. A recording spectrophotometer was used (Type CF4DR, Optica U.K., Gateshead-on-Tyne, England).

Emission spectroscopy. We are indebted to our colleagues Mr L. C. Thomas and Mr J. L. Clipson for these determinations.

Infrared spectroscopy. We are indebted to our colleague Dr K. Norris for this.

Haematocrit, plasma protein and plasma inorganic phosphate. These were determined by the methods described by Smith *et al.* (1955*b*).

Crude anthrax toxin produced in vivo. This was as described by Smith *et al.* (1955*a*).

Crude anthrax toxin produced in vitro. This was prepared by the method of Thorne, Molnar & Strange (1960) and horse serum (10 %, v/v) was added before filtration.

Crude factor I from toxin produced in vitro. A modification of the method of Thorne *et al.* (1960) was used. A culture (1 l.) containing crude antigen of *Bacillus anthracis* (Thorne & Belton, 1957; Strange & Thorne 1958) was passed through a sintered-glass bacterial filter (6 cm. diameter; 5/3 porosity), which was washed with 0.05M-phosphate buffer (pH 7; 4 × 20 ml.) at 0–2°. On extraction of the filter for 1 hr. with ice-cold saturated Na₂CO₃ solution (4 ml.) followed by neutralization of the extract with HCl a solution of crude factor I was obtained. One batch of culture contained 40–100 l. and 40–100 filters were washed and extracted within 2 hr. of filtration of the original culture.

Crude factor II from the toxin produced in vitro. This was precipitated from the filtrate of the culture described above, by ammonium sulphate as described by Strange & Thorne (1958) and dialysed for 6 hr. at 0–2° against buffered saline (0.01 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris buffer) in saline; pH 7.4).

Purified preparation of factor II of the anthrax toxin produced in vitro. This was the preparation of Strange & Thorne (1958) described by Sargeant *et al.* (1960).

RESULTS

Purification and properties of factor I of the anthrax toxin

Purification of factor I on diethylaminoethyl cellulose (DEAE-C). A solution of crude factor I (200 ml. ≡ 50 l. original culture; factor I null point 1/500; protein content about 0.06 %) at pH 7.4 was diluted with 0.005 μ phosphate buffer (1800 ml.; pH 7.4) and applied to a column (4.5 cm. diameter, 10 cm. length) of DEAE-C (25 g.) which had been packed at 4 lb./sq.in. pressure and equilibrated with 0.1 μ phosphate buffer (pH 7.4). The flow rate was adjusted to 20 ml./min. by application of a slight positive pressure. The effluent (2000 ml.) had negligible activity and the column was eluted successively with quantities (450 ml.) of 0.1, 0.12, 0.15, 0.17 and 0.2 μ phosphate buffers (pH 7.4). In preliminary experiments each application of buffer was collected as a single fraction (450 ml.); the bulk (60–90 %) of the factor I activity was eluted by the 0.15 and 0.17 μ buffers.

In subsequent experiments the eluates from the applications of the 0.15 and 0.17 μ buffers were each collected in 6 × 75 ml. fractions. Each fraction was purified and concentrated in the following manner. Each fraction (75 ml.) was diluted with distilled water (225 ml.) and applied to a column (2 cm. diameter, 1 cm. length) of DEAE-C (0.2 g.) equilibrated with 0.1 μ phosphate buffer (pH 7.4). No pressure was applied and the flow rate was 5–10 ml./min. The effluent (300 ml.) contained only a small amount of factor I activity but over 75 % of the original material reacting as protein in the method of Lowry *et al.* (1951). The column was washed

once with 0.1μ phosphate buffer (6 ml.; pH 7.4) and the active material eluted in 0.2μ phosphate buffer (pH 7.2) containing M/5 NaCl (4 ml.).

Before combining any active fractions for further processing the 12 concentrated fractions from the eluates with 0.15 and 0.17μ buffers were examined separately on serological diffusion plates for the presence of impurities. Preliminary work showed that factor I was associated with a line formed on serological precipitation plates against 'spore' anthrax antiserum (H. 533), whereas it formed no line against 'antigen' antiserum (H25) (see Sargeant *et al.* 1960). On serological diffusion plates the 12 concentrated fractions formed against anthrax antisera faint extraneous lines in a pattern which varied slightly from batch to batch but was usually as indicated in Table 1.

Table 1. *Pattern of lines formed on serological diffusion plates of toxin fractions from DEAE-C columns with anthrax antiserum*

Vertical braces enclose fractions usually bulked for final concentration.

Buffer used for elution	Fraction*	Intensity of line associated with factor I†	Intensities of lines (1) (2) and (3) formed by extraneous antigen†				
			Antiserum H 533		Antiserum H 25		
			(1)	(2)	(1)	(2)	(3)
0.15 μ phosphate (pH 7.4)	1	1	1	.	1	1	.
	2	3	1	.	1	1	.
0.17 μ phosphate (pH 7.4)	3	5	1	.	1	1	.
	4	4	.	.	.	1	.
	5	4	.	.	.	1	.
	6	4	.	.	.	1	.
	7	3	.	.	.	1	.
	8	3	.	.	.	1	.
	9	3	.	.	.	1	.
	10	3	.	1	.	1	1
	11	3	.	1	.	1	1
	12	2	.	1	.	1	1

* 75 ml. concentrated to 4 ml. on a 2nd DEAE-C column (see text).

† Numbers 1-5 indicate approximately the intensity of lines on serological diffusion plates (high number; high intensity).

Appropriate fractions (usually 3-8, but in each experiment the inclusion of fractions 2, 9 and 10 or exclusion of fractions 3, 7 and 8, depending on the particular pattern of lines formed by the fractions on serological diffusion plates) from this and a second similarly fractionated batch (200 ml.) of crude factor I, were combined, dialysed overnight at $0-2^\circ$ against 0.02μ phosphate buffer (pH 7.4) and applied to a column (0.5 cm. diameter, 3 cm. long) of DEAE-C (80 mg.) equilibrated with 0.02μ phosphate buffer (pH 7.4). The effluent (about 50 ml.) contained negligible activity. The column was eluted with 0.2μ phosphate buffer (pH 7.2) containing M/5 NaCl. The first 0.4 ml. of eluate, which contained negligible activity, was discarded. The next 0.8 ml. constituted the final preparation of factor I which was kept at -20° until required.

Yield and recovery of activity. In five similar experiments, 0.8 ml. of final product having an average factor I null point of 1/64,000, containing about 0.4-0.7% of

non-diffusible material and 0.3–0.5 % of 'protein' (see Methods), was obtained from 400 ml. of crude factor I having an average factor I null point 1/500 and containing 0.06 % of protein (see Methods). Hence about 25 % of the original factor I activity was recovered in the final product which had been considerably purified from other protein. There was no extensive loss of activity during the process since, in all the fractions taken therefrom, 80 % of the original activity was recovered.

For most experiments, the final product was used as prepared in solution and stored at -20° . For chemical analysis the solution was dialysed for 7 days at $0-2^{\circ}$ against frequent changes of distilled water until free from salt and then freeze-dried. After this prolonged dialysis 50 % of the original activity was lost; after freeze-drying, the material was not completely soluble.

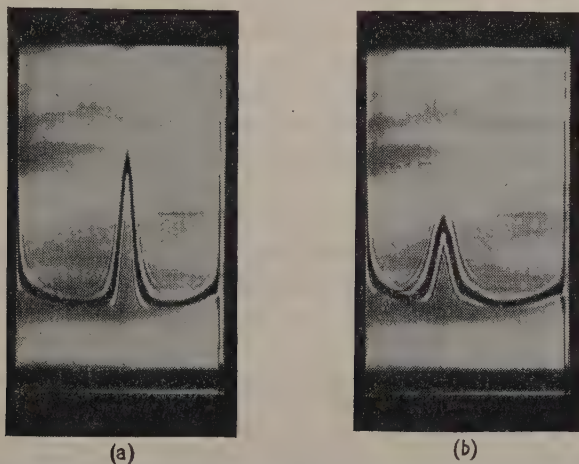


Fig. 1. Ultracentrifuge diagrams of the final preparation of anthrax toxin factor I. Concentration about 0.75 % (w/v), 0.2μ phosphate buffer (pH 7.4); field 100,000 g. Pictures taken (a) 8 min. and (b) 32 min. after sedimentation in synthetic boundary cell (sedimentation right to left).

Criteria of purity

Ultracentrifugation. The final preparation (0.7 %) showed one major component when examined in a synthetic boundary cell at pH 7.4 in 0.2μ phosphate buffer (see Fig. 1).

Paper electrophoresis. The final preparation was concentrated by dialysis against carbowax. The material (0.3 mg.) showed no evidence of heterogeneity when examined in 0.2μ barbitone buffer (pH 8.6) and 0.2μ acetate buffer (pH 5); it was almost stationary but it did move from the origin, towards the anode at pH 8.6 and towards the cathode at pH 5.

Serological precipitation in gel diffusion plates. Experiments described in an addendum to this paper indicated that two extraneous serologically-reacting materials were present in the final product, but that the degree of contamination was not more than 0.5 % of one material and 5 % of the other.

Biological properties

Oedema production. In the assay for factor I activity about 0.02 μ g. produced an oedematous reaction when mixed with the standard quantity of factor II, but 6 μ g. produced no reaction when injected alone. The material was stable at -20° at pH 7 for 1–2 months, but at $0-2^{\circ}$ 50 % of factor I activity disappeared after 7 days at pH 7, and 75 % was lost after 7 days at pH 5 and 9. The material (in 0.2 μ phosphate buffer, pH 7.4) lost 50 % of its activity in 2 hr. at 37° , 75 % of its activity when shaken with glass beads for 1 hr. at 0° and all of its activity on standing for 24 hr. at 0° with cysteine (0.2 %), sodium metabisulphite (0.1 %) and potassium periodate (0.05 %).

Lethality to mice (see below)

Serological precipitation in gels. The behaviour of the final preparation of factor I was described by Sargeant *et al.* (1960). The line associated with factor I activity was just formed by 0.5 μ g. of the final preparation in serological diffusion plates against 'spore' anthrax antiserum (H 533); no line was formed against 'antigen' anthrax antiserum (H25) unless the material was examined at high concentrations (see above).

Immunizing activity. In the assay for immunizing activity in guinea pigs 120 μ g. of the final preparation showed no significant activity. Similarly, 40 μ g. or 100 μ g. of the final preparation did not immunize rabbits (10 rabbits were used in each batch and the material was mixed with horse serum before injection).

Table 2. *Chemical analysis of the final preparation of anthrax toxin factor I*

	%
N	12.0 (Kj), 12.0 (D)
Carbohydrate	6.4 (as glucose)
Lipid	Less than 3
Hexosamine	Less than 1
P	0.7
Ash (SO_4)	10.1*
Amino acids	All usual ones and no evidence of extra ones

* After reheating with hydrofluoric acid, 10.3 %.

Chemical properties

Table 2 summarizes the chemical analyses of the final product. Fig. 2 shows its infrared spectrum and Fig. 3 (curve A) its ultraviolet absorption spectrum in neutral solution. The material was not solely protein, although it contained much amino acid material; it had no significant amount of lipid and some carbohydrate residues. The most striking aspect of its chemical analysis was a high metal content as shown by its high ash, and an apparent nucleic acid content (about 6–8 % from its absorption at 260 $m\mu$) and its P content. These aspects were investigated in more detail.

The metal content. The high ash of the final material seemed to be a specific property and not due to the fractionation process. Thus, ovalbumin, rabbit γ -globulin, human serum albumin and two samples of factor II of anthrax toxin had 1.3, 2.7, 0.9, 0.6 and 1.2 % sulphate dashes, respectively, after being subjected to the same

fractionation process as that described above for factor I. The high ash was not due to the presence of SiO_2 (or B_2O_3) since heating with HF did not decrease it.

The nature of the metal content was investigated by spectroscopic analysis. Mg, Ca, Ni and Cu were present in appreciable amounts in the four batches of material examined; only traces of other metals were present. The estimated contents (which were subject to an error of $\pm 100\%$ due to the small amount of material examined and the necessity of estimating all metals on one sample) of the four batches fell in the ranges (%): Mg, 0.001–0.1; Ca, 0.003–0.8; Ni, 0.3–0.75; Cu, 0.1–1.3; but the relative proportions were not consistent from batch to batch.

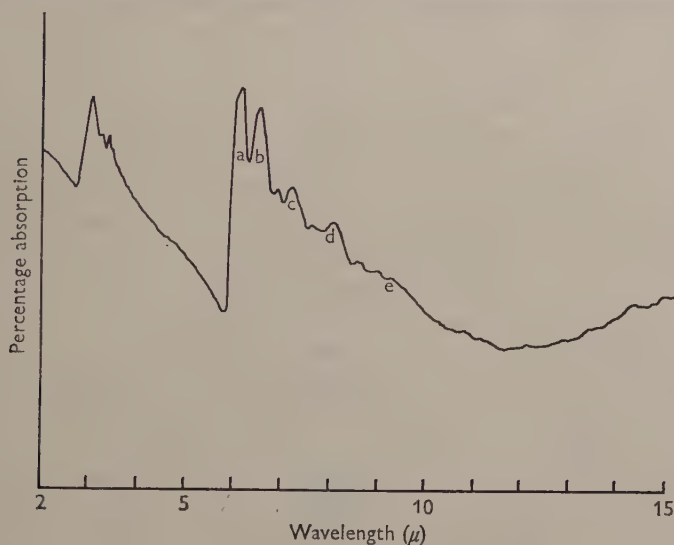


Fig. 2. Infrared spectrum of the final preparation of anthrax toxin factor I; 0.8 mg. on AgCl. Peaks *a* and *b* indicate protein; peak *c*, $-\text{CH}_2-$ and $-\text{CH}_3$ groups; peak *d*, $\text{P}=\text{O}$ groups; the absorption *e* a small amount of carbohydrate.

In attempts to remove the metal content or at least to obtain a consistent pattern, four batches of active material were prepared as described above but with the use of deionized water throughout the process and DEAE-C columns which had been pretreated with ethylenediaminetetraacetic acid (EDTA) to remove metals as follows: 100 g. DEAE-C were treated with 4 l. 0.1% EDTA overnight, decanted, re-treated with a further 4 l. 0.1% EDTA and washed with 3×4 l. 0.1M phosphate buffer (pH 7.4). The sulphated ash of the final material (11.3 and 12.6% on two of the batches) was not decreased by these precautions, although the nature of the metal content changed to some extent; the Ni and Cu content appeared to be decreased (range Ni 0.12–0.19%; Cu 0.1–0.5%) and the Mg and Ca content increased (range Mg 0.17–1.1%; Ca 0.3–1.0%). It appeared that the preparation of factor I had a chelating action on most metals and that no single metal was characteristic of the preparation.

The absence of RNA and DNA. The following observations were carried out in collaboration with our colleague Mr H. E. Wade. (1) Adequate control experiments showed that the absorption of u.v. radiation was not due to the high Ni and Cu content. (2) Although in neutral solution the u.v. absorption spectrum of the

final preparation of factor I (Fig. 3, curve A) resembled the spectrum of a mixture of 10% yeast RNA in ovalbumin (Fig. 3, curve B), the shifts in their spectra (Fig. 3, curves C and D, respectively) in 0.3N-KOH were different. The absorption of the experimental sample at 260 $m\mu$ was less than that at neutral pH, whereas the absorption of the artificial mixture of nucleic acid + protein was greater than at neutral pH. Furthermore, in alkali the experimental sample showed a characteristic plateau of absorption at 270–295 $m\mu$ which was not shown by the control mixture. (3) The final preparation of factor I (2 mg.) and the control mixture (2 mg.) were treated separately as follows. A solution in 0.3 N-KOH (3 ml.) was left at 37° for 18 hr. and cooled to room temperature. Perchloric acid solution (0.1 ml., 72%, w/v)

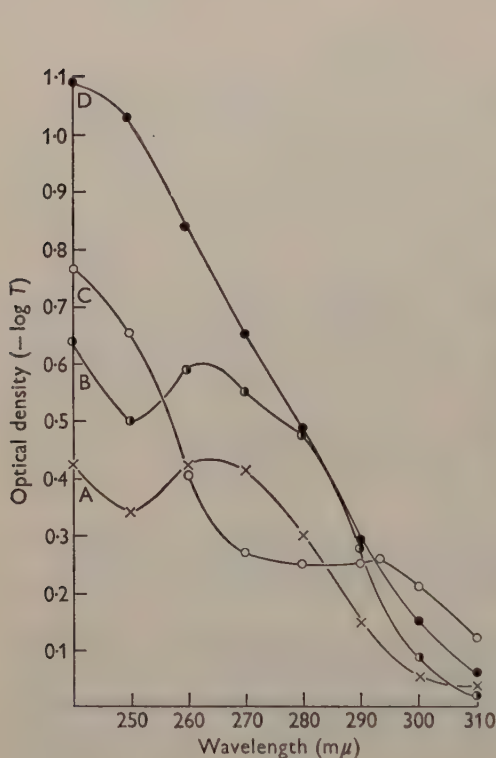


Fig. 3

Fig. 3. Ultraviolet absorption spectra of the final preparation of anthrax toxin factor I and ovalbumin containing 10% (w/w) yeast RNA. Adsorption determinations were made in a 0.5 cm. cell. Curve A: 0.05% final preparation of factor I at pH 7.4. Curve C: 0.05% final preparation of factor I in 0.3 N-KOH. Curve B: 0.05% of a mixture of ovalbumin with 10% yeast RNA at pH 7.4. Curve D: 0.05% of a mixture of ovalbumin with 10% yeast RNA in 0.3 N-KOH.

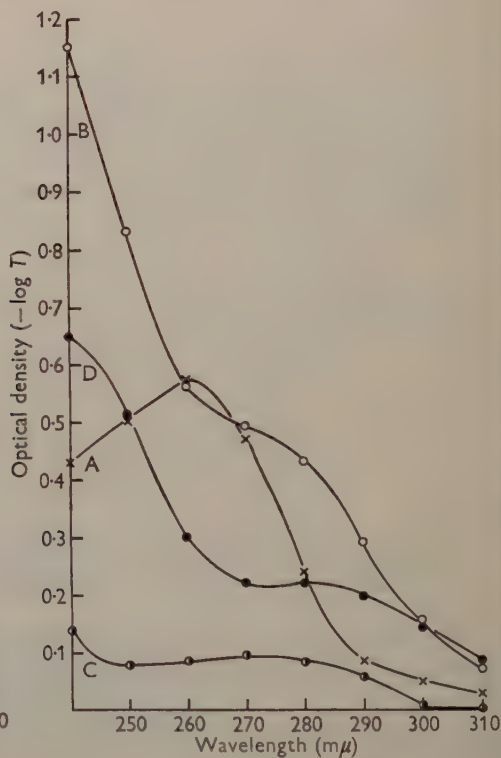


Fig. 4

Fig. 4. Ultraviolet absorption spectra of the products of hydrolysis of the final preparation of factor I. Adsorption determinations were made in a 1 cm. cell (compare Fig. 3). Original material (2 mg.) was hydrolysed in a volume of 3 ml. (see text); all materials were adjusted to this volume for examination. Curve A: extract from mild alkaline hydrolysis examined in acid solution (0.1 N-HClO₄). Curve B: extract from mild alkaline hydrolysis examined in alkaline solution (0.1 N-KOH). Curve C: extract prepared by acid hydrolysis of the residue left after alkaline hydrolysis; examined in acid solution. Curve D: residue left after alkaline and acid hydrolysis; examined in neutral solution.

was added and the precipitate removed by centrifugation and reserved (see below); the extract was clarified by adding ovalbumin solution (0.1 ml., 0.2 %) and recentrifugation. The spectra of the extracts, which should contain all the ribonucleotides from RNA if present, were examined under acid (as prepared) and alkaline (after adding 20 N-KOH to make the solution 0.1 N-KOH and removing the KClO_4 by centrifugation) conditions. The spectra of the extracts from the control mixture containing RNA were as expected; practically all the original material absorbing at 260 $\text{m}\mu$ was soluble in acid after alkaline hydrolysis and the extinction ($-\log T$) of the extract at 240 $\text{m}\mu$ was less than that at 260 $\text{m}\mu$ under both acid and alkaline conditions. In contrast, the spectra of the extract from the experimental sample [Fig. 4, curves A (acid conditions) and B (alkaline conditions)] showed that much of the material in the original sample which absorbed at 260 $\text{m}\mu$ had not been liberated by alkaline hydrolysis (compare Fig. 3, curve C with Fig. 4, curve B) and that the extracted material was not the usual mixture of ribonucleotides, i.e. there was a large increase of absorption at 240 $\text{m}\mu$ (relative to that at 260 $\text{m}\mu$) when the conditions changed from acid to alkaline. The absence of ribonucleotides in this extracted material was confirmed by paper ionophoresis (Wade, 1961). The hydrolysate was neutralized with HClO_4 and concentrated in a desiccator before applying to the paper. A comparison with a similar extract from the control mixture of RNA + protein indicated that the original sample of factor I contained less than 0.6 % RNA. (4) The absence of DNA was indicated by the following hydrolytic studies. The material precipitated by HClO_4 after alkaline hydrolysis of the final preparation of factor I, including the deposit centrifuged after clarification with ovalbumin, was suspended in water (3 ml.) and heated with HClO_4 solution (0.15 ml., 72 %, w/v) for 15 min. at 90°. A deposit was centrifuged from the cooled product and the spectrum of the supernatant fluid examined (Fig. 4, curve C); it showed no evidence for the presence of hydrolytic products of DNA. On the other hand, when the deposit from this acid hydrolysis was dissolved in water (3 ml.) at pH 7 (addition of 5 N-NaOH), its spectrum showed that it still retained material absorbing at 260 and 240 $\text{m}\mu$ (Fig. 4, curve D). (5) In a micro-adaptation of the diphenylamine method for deoxysugars (Volkin & Cohn, 1954) no colour was formed by the final preparation of factor I (90 $\mu\text{g.}$), whereas 2.5 $\mu\text{g.}$ of DNA (thymus) produced a colour.

Recognition of a third factor of the anthrax toxin

The low toxicity for mice of oedema-producing mixtures of purified factors I and II. The results in the top half of Table 3 show that the final preparation of factor I and a purified preparation of factor II produced no oedema in rabbits and did not kill mice when large amounts were injected alone, but that smaller quantities injected together produced large skin reactions in rabbits and killed some mice. However, the lethality of these mixtures for mice was much smaller than that warranted by their capacity for oedema production when compared with results of similar experiments with crude preparations (see bottom half Table 3). Thus, crude unseparated anthrax toxin produced *in vivo* and *in vitro* killed more mice at concentrations which produced a relatively smaller amount of oedema in rabbits; and the same type of result was obtained by injecting mixtures of crude factors I and II which, like the purified factors, did not when injected alone produce oedema in rabbits and were almost non-toxic for mice. The finding that some factor which con-

tributed to lethality for mice was present in either crude factor I or II and absent from the purified preparation, was indicated by the results shown in the last two lines of Table 3. In contrast to mixtures of purified I and II, mixtures of either crude I + purified II or purified I + crude II killed mice, although they produced a relatively small skin reaction in rabbits.

Table 3. *Oedema production and lethality for mice of mixtures of crude and purified factors I and II of the anthrax toxin*

Results are the average or aggregate of similar results on several batches of material. In all cases the production of oedema and the killing of mice were completely neutralized by mixing the solutions with 'spore' (H533) anthrax antiserum (1/3 vol.).

Material injected	Approx. amount* (μ g.) in 0.5 ml.		Size of skin reaction† (0.2 ml.)	Lethality for mice (0.5 ml.) dead/total
	Factor I	Factor II		
A. Final prep. of factor I	16	—	Nil	0/21
B. Purified factor II	—	320	Nil	0/14
A+B	8	80	17:75†	19/31
A+B	4	40	15:62	5/86
A+B	2	20	15:60	0/15
A+B	1	10	14:40	0/5
A+B	0.5	5	11:40	0/5
Crude anthrax toxin (<i>in vivo</i>)	—	—	9:40	64/75
Crude anthrax toxin (<i>in vitro</i>)	—	—	10:40	15/30
C. Crude factor I	240	—	Nil	0/22
D. Crude factor II	—	600	Nil	4/14
	—	300	Nil	3/25
	—	150	Nil	1/20
	—	75	Nil	0/10
C+D	60	75	8:35	32/35
A+D	4	75	9:40	17/27
C+B	60	40	9:37	25/30

* The amounts of materials equiv. about 500 times the quantities corresponding to the null points in the assays for factors I and II (see methods) were (1) final preparations of factor I 4 μ g. (2) purified factor II 80 μ g.; (3) crude factor I 60 μ g. ('protein'); (4) crude factor II 150 μ g. ('protein').

† A skin reaction of size 17:75 means that the oedematous lesion had a fold thickness of 17 mm. and a diameter of 75 mm. Significant differences were 2 mm. in fold thickness or 10 mm. in diameter; these differences were produced by 2-4-fold differences in toxin concentrations.

Enhancement of the lethality for mice and oedema production in rabbits of mixtures of purified factors I and II by adding serum and other proteins. The possibility that the effect of the constituents of the crude preparations described above was a non-specific action of extraneous protein (e.g. possibly by protecting the toxin against destruction in the mouse) was investigated. The results in Table 4 show that the specific lethality for mice of mixtures of the final preparation of factor I + purified factor II was significantly increased by adding various sera and proteins. However, the addition of this extraneous protein also increased the skin reactions; the increase was about 2-4-fold when compared by the more sensitive null point assay, and the anomaly described above regarding size and skin reaction and lethality for mice

remained. This effect of serum and other proteins only occurred with mixtures of the purified factors; in similar experiments with crude preparations extraneous proteins had little or no effect on oedema production or on lethality for mice.

Fractionation of a third factor of the anthrax toxin. A factor was sought which, when added to a mixture of purified factors I and II, would form a preparation which killed mice at concentrations that produced in rabbits a relatively small skin reaction. Preliminary experiments showed that such a factor was present in fractions collected from the chromatography of crude factor I on DEAE-C when the column was eluted with 0.2μ phosphate buffer after factor I had been removed with more dilute buffers (see previously). The factor was also present in those fractions discarded during the purification of factor II by the method of Strange & Thorne (1958). A preparation of this factor III was produced for the experiments described below by the following method.

Table 4. *Effect of serum and other proteins on oedema production and lethal effect of mixtures of purified anthrax toxin factors I and II*

The lethal and oedema-producing effects were completely neutralized when horse anthrax antiserum (spore H533) was used instead of the other sera.

Material in 0.5 ml.

Purified factor I (μ g.)	Purified factor II (μ g.)	Serum or other protein * (0.2 ml.)	Lethality for mice (0.5 ml.): dead/total	Skin reactions in rabbits (0.2 ml.)	
				Size†	Null point‡
4	40	Saline	5/86	15:62	1/320
4	40	Horse serum	22/25	17:62	1/1280
4	40	Rabbit serum	18/30	18:60	1/1280
4	40	Guinea-pig serum	8/10	17:65	1/1280
4	40	Ovalbumin (5%)	4/5	16:58	1/640
4	40	Sodium polyglutamate (1%)	4/5	15:64	1/640
2	20	Saline	0/15	—	—
2	20	Horse serum	4/25	—	—
2	20	Rabbit serum	2/5	—	—
2	20	Guinea-pig serum	3/10	—	—
2	20	Ovalbumin (5%)	0/5	—	—
2	20	Sodium polyglutamate (1%)	0/5	—	—

* When injected alone these preparations did not kill mice (0.5 ml.) or evoke oedema (0.2 ml.) in rabbits.

† See Table 3.

‡ The null point was the first dilution of two fold descending dilutions which did not form an oedematous skin reaction.

Filtrate (5 l.) from a culture containing crude anthrax toxin (see Methods) was applied to a column (4.5 cm. diam.; 4 cm. long) of DEAE-C (5 g.) which had been equilibrated with 0.1μ phosphate buffer (pH 7.4). No pressure was applied and the flow rate was about 30 ml./min. Factor III was retained on the column and most of the factor II was not.

The column was eluted with: (1) 0.15μ phosphate buffer (60 ml.; pH 7.4); (2) 0.2μ phosphate buffer (60 ml.; pH 7.4); (3) 0.2μ phosphate buffer containing $0.05M$ -NaCl (60 ml.; pH 7.4); (4) 0.2μ phosphate containing $0.2M$ -NaCl (180 ml.; pH 7.2). Factor III was eluted by buffer (4); 15 ml. fractions of this were collected, assayed for protein and appropriate fractions corresponding with the elution peak

were concentrated in the following manner. The bulked fractions from 5 batches of filtrate were dialysed against 0.1μ phosphate buffer (pH 7.4, $0-2^\circ$, overnight) and applied to a column (2 cm. diam.; 1 cm. length) of DEAE-C (0.2 g.) which had been equilibrated with 0.1μ phosphate buffer (pH 7.4). No pressure was applied and the flow rate was 5–10 ml./min. Factor III was eluted with 0.2μ phosphate buffer containing 0.2 M-NaCl (8 ml.). This preparation of factor III contained 0.15% protein. In gel diffusion plates against 'spore' antiserum (H533) it ($10\mu\text{g.}$) formed two lines which were also formed by crude anthrax toxin produced *in vivo* and by an impure preparation of factor I from this source (see Stanley *et al.* 1960); these lines were different from those formed by the final preparations of factor I (see above) and of purified factor II (see Sargeant *et al.* 1960). Against 'antigen' (H25) antiserum the preparation of factor III formed a line which very easily dispersed and dissolved in antigen excess, indicating a low content of factor III antibody in this antiserum.

Table 5. *Effect of partially purified third factor (III) of the anthrax toxin on oedema production and lethal effect of mixtures of factors I and II*

Material in 0.5 ml.				
Purified factor I ($\mu\text{g.}$)	Purified factor II ($\mu\text{g.}$)	Partially purified factor III* ($\mu\text{g.}$)	Lethality for mice (0.5 ml.): dead/total	Size of skin reaction in rabbits† (0.2 ml.)
8	80	—	19/31‡	17:75‡
4	40	—	5/86	15:62
2	20	—	0/15	15:60
16	—	16	0/10	Nil
4	—	8	0/10	Nil
4	—	4	0/10	Nil
4	—	2	0/20	Nil
2	—	8	0/20	Nil
—	160	16	14/19	Nil
—	40	16	28/30	Nil
—	40	8	28/69‡	Nil
—	40	4	6/49	Nil
—	40	2	0/30	Nil
—	20	8	3/20	Nil
4	40	8	48/70‡	8:35‡
4	40	4	19/50	10:45
4	40	2	4/30	15:60
2	20	8	8/15	6:27

* When injected alone partially purified factor III—64, 32, 16 and $8\mu\text{g.}$ —killed 0/10, 0/10, 0/15, 0/10 mice respectively and produced no oedema in the skin of rabbits.

† See Table 3.

‡ These lethal and oedema producing effects were completely neutralized in adequate batches (10–20) of animals by admixture with anthrax antiserum ('spore' H533)— $\frac{1}{3}$ rd vol.

The results shown in Table 5 indicate that: (1) In common with factors I and II, factor III was non-lethal for mice and produced no oedema in rabbits when injected alone in large amount. (2) A mixture of factors I and III at high concentrations was non-lethal for mice and formed no skin reactions in rabbits. (3) In common with a mixture of factors I and III, a mixture of factors II and III showed synergic

action and was toxic for mice. On the other hand, it did not produce oedema in the skin of rabbits as did the I+III mixture. (4) A mixture of factors I, II and III showed synergic action in toxicity tests in mice and produced a greater effect than expected from the addition of the effects of the pairs of factors described in (3). Table 6 shows the statistical analysis of the cogent results, for which we are indebted to our colleague Mr S. Peto. (5) The addition of factor III to a mixture of factors I and II decreased the size of the oedematous skin reactions produced in rabbits. (6) All these toxic effects were neutralized by anthrax antiserum.

Injection of a mixture of factors I, II and III (about 10 and 20 times the amounts which killed mice) killed guinea pigs (see Table 7). This toxicity was neutralized by anthrax antiserum.

Table 6. *Synergism of factors I, II and III of the anthrax toxin in mice*

Doses* (μ g.)			Dead (%)* obtained when submitted to				Dead (%) expected assuming independent action	Significance of difference		
I	II	III	I+III	I+II	II+III	I+II+III		t	Prob. %	Comment
4	40	8	Nil	5.8	40.6	68.6 (5.5)†	52.0 (6.8)†	3.0	0.3	Highly sign.
4	40	4	Nil	5.8	12.2	38.0 (6.9)	17.3 (4.9)	2.4	1.5	Sign.
4	40	2	Nil	5.8	1.5‡	13.3 (6.2)	7.2 (3.2)	0.9	37	Not sign.§
2	20	8	Nil	2.5‡	15	53.3 (12.9)	17.1 (8.3)	2.3	1.9	Sign.

* Obtained from data in Table 5. † Standard deviations. ‡ Assuming 1/2 animal died.

§ Note small sample sizes.

Table 7. *The toxicity of a mixture of anthrax toxin factors I, II and III in guinea pigs (250 g.)*

Doses (μ g.) of factors			Antiserum*	Dead/total
I	II	III		
80	600	160	Nil	2/2
40	300	80	Nil	5/6
40	300	80	0.8 ml.	0/5

Injections (2.5 ml.) were made into the saphenous vein (Smith *et al.* 1955*a*).

* 'Spore' anthrax antiserum (H533).

Observations were made during the terminal phase on two of the guinea pigs (250–300 g.) killed by the mixtures of factors I, II and III. For comparison corresponding observations were made on a few normal animals; these were the same as those described by Smith *et al.* (1955*a*, *b*). The two animals died with the clinical signs of shock; their bleeding volumes (Smith *et al.* 1955*a*, *b*) were low (about 4 ml.) and their plasma inorganic phosphate and non-protein-N contents were high (PO_4^{--} , 10.5, 15.5; non-protein-N, 100, 85 mg./100 ml.). Evidence that oligæmia contributed to the shock syndrome in these guinea pigs (compare guinea pigs dying of anthrax and from injection of crude anthrax toxin produced *in vivo*; see Smith *et al.* 1955*a*, *b*) was provided by the fact that their haematocrit values were high (about 50 %) and their plasma protein contents low (500 and 475 mg. N/100 ml.). One guinea pig had a gross subcutaneous oedema comparable with that seen when some but not all guinea pigs die from anthrax.

DISCUSSION

In any comparison between the final preparations of factor I of the anthrax toxin produced *in vitro* (as described here) and that produced *in vivo* (Stanley *et al.* 1960, and subsequent unpublished observations), it must be remembered that whereas the former preparation was relatively pure, the latter was known to contain some constituents of guinea pig plasma (about 15 %) and to be serologically heterogeneous in gel diffusion against anthrax antisera. Bearing this in mind, the two preparations resembled each other in being unstable, having a high ash content, absorbing light at 260 m μ , being almost stationary when electrophoresed on paper at pH 8.6, containing much protein but having a relatively low N content, and having a small carbohydrate and phosphorus content (the latter might conceivably be due to a strong binding of phosphate from the buffers used in the process). The two preparations were quite different in three respects, however: (1) The preparation from the *in vivo* source tended to aggregate and sediment more rapidly in the ultracentrifuge than that from the *in vitro* source. (2) The preparation from the *in vivo* source contained much lipid (probably serum lipid) whereas the other preparation did not. (3) The preparation from the *in vivo* source immunized animals, whereas the present preparation did not.

The most striking chemical property of factor I of the anthrax toxin was its high and complete metal content, the nature of which was not consistent from batch to batch. Factor I was not an acid, binding the metals by a purely ionic linkage, because it did not migrate rapidly when electrophoresed on paper at pH 8.6. It appeared therefore that factor I had a strong and indiscriminate chelating action on the metals which were present in the original culture medium (this contained CaCl₂, MgSO₄ and 'Casamino Acids' with a high ash) and in materials (e.g. DEAE-C) used for purification. The chelating action might be due to the presence of the chemical group which absorbed light at 260 m μ and which was not RNA or DNA; this might be the same group that produced in alkaline solution a characteristic 'blip' in the u.v. absorption at about 293 m μ . The nature of this group is at present unknown; the main difficulty in its investigation is the very small amount of material available for experiment. The metal-binding activity of factor I may be the reason for its biological action but this is not proven; a 0.1 % (w/v) solution of EDTA did not replace factor I in the biological assays.

A comparison of the nature of the biological properties of a mixture of final preparation of factor I + purified factor II with those of crude materials led to the demonstration of a third factor of the anthrax toxin. This factor was partially purified from crude factor II produced *in vitro*, and shown to be different from factors I and II and present in the anthrax toxin produced *in vivo* by serological methods. Table 8 summarizes the biological relationship between the three factors. The reason for the decrease in skin reaction when factors I and II were mixed with factor III is not clear. Possibly factor III helps in the dispersion of the other factors and leads to a smaller local action. In contrast to the action of factor III, the addition of serum to factors I and II increased their capacity for local oedema production as well as the mouse lethality, probably by a non-specific protective effect. Although it was shown that mixtures of factors I, II and III showed synergism in mouse toxicity tests, it was impossible to obtain the optimal proportions of the three

factors because of the large amount of material and the number of mice needed for a comprehensive titration. For the same reason, although it was shown that guinea pigs were killed specifically by injecting a mixture of the three factors, the determination of their optimal proportions and the demonstration of synergism in guinea pigs was precluded. The guinea pigs killed by the mixtures of the three factors died with some of the symptoms of oligæmic secondary shock as did guinea pigs dying of infection (Smith *et al.* 1955*b*).

Table 8. *Relationship in toxicity tests of the I, II and III factors of the anthrax toxin*

All toxic effects were neutralized by anthrax antiserum

Factors	Oedema production in skin of rabbits	Lethality for mice
I	Nil	Nil
II	Nil	Nil
III	Nil	Nil
I + II	+++	+
I + III	Nil	Nil
II + III	Nil	++
I + II + III	++	++++
Crude toxin	++	++++

The existence of a third factor of the anthrax toxin possessing the properties described above explains an apparent anomaly in previous results (see Smith *et al.* 1956, Table 2, line 3) when the injection of a mixture of crude *in vitro* antigen + crude factor II prepared *in vivo* killed mice, but did not produce a large skin reaction. The demonstration of three factors of the anthrax toxin leads to speculation as to whether at one time they were joined, at least in a loose complex, and to whether mixtures of the factors would immunize animals better than any individual factor.

Our thanks are due to Mr F. C. Belton for producing the anthrax toxin *in vitro* and to Mr R. Blake for excellent technical assistance.

NOTE ON SEROLOGICAL PRECIPITATION IN GELS AS A CRITERION OF PURITY OF ANTIGENS

By H. SMITH, K. SARGEANT and J. L. STANLEY

In the purification of factors I and II of the toxin of *Bacillus anthracis* (Stanley *et al.* 1960; Sargeant *et al.* 1960) serological precipitation in gels was used as a criterion of purity of antigens. This focused our attention on a problem which we think is evaded in similar studies on bacterial and other products and which warrants some discussion.

A purified product may be obtained which shows no evidence of heterogeneity when examined ultracentrifugally or electrophoretically in the conventional manner. At an arbitrarily chosen concentration, the product may form one major non-

composite (cf. Sargeant *et al.* 1960) line in serological diffusion plates against a strong antiserum. However, this same antiserum may detect many antigenic impurities since it is prepared by hyperimmunizing animals with either the original mixture of antigens, or with the appropriate live organism. Hence, when higher (and occasionally lower) concentrations of the purified product are diffused against this antiserum further faint lines may appear, indicating the presence of antigens in addition to the one forming the major line; these additional antigens can usually be demonstrated in other fractions discarded during the separation of the final product. The difficulty is to know how one can be reasonably certain that the antigens which produce the additional lines are not present in the purified product in large amounts. The fact that, at the concentration examined, these additional lines are fainter than the major line does not necessarily mean that the antigens producing them do not predominate in the product under examination. The antigens in question might be present in large amounts but precipitated feebly in the test system, e.g. they might be poor antigens and hence the serum relatively deficient with their precipitating antibodies. When the serological behaviour of products on gel-diffusion plates is described there is usually no mention of the concentrations at which the material first formed one line and then more lines. Furthermore, if one line were formed, no assessment is made of the purity of the product on the basis of relative weights of the precipitating antigens.

We propose a practical working rule for studies of this kind which, although not foolproof, sets a standard of assessment of impurity on a weight basis comparable with the conventional methods of analytical electrophoresis and ultracentrifugation. Usually in the latter procedures a full examination of the product does not take place because of the large effort involved and especially because of the lack of sufficient material. As a compromise, the material is generally examined at one or two pH values at concentrations near 1%, w/v. Such examinations can indicate that the product is not grossly heterogeneous but they will not detect less than 5% of impurity. The object of the following procedure with the gel-diffusion method is to make reasonably certain that extraneous antigens, which are usually more easily detected by this method than by other methods, do not exceed about 5% of the final product.

The final product is examined in one of the conventional systems of gel diffusion. This should be accurately described and the antiserum used should be the one which will detect the largest number of antigenic impurities. Concentrations of 0.5–1% to 0.001% are examined for the production of extraneous lines (cf. Sargeant *et al.* 1960) and then the importance of these lines is assessed by titrations. It is assumed that the line-forming ability of any antigen does not vary in different preparations.

The determination by titration in gel-diffusion plates of the amount of extraneous antigens in a purified product would be a simple matter if pure preparations of these antigens were available for comparison; but usually this is not so, since the extraneous antigens are unknown and may be of no importance except as impurities in the final product. However, if we are aiming at the standard of assessment of impurities described above, quite crude samples of extraneous antigens are suitable for comparative titrations. The aim is to obtain a discarded fraction from the purification process, which in a gel-diffusion system forms a line at a concentration about

20 times less than the lowest concentration of purified preparation which forms this same line against the same antiserum in the same system. If this can be achieved, it means the purified product contains less than about 5% of the extraneous antigen. This contamination may be far less than about 5%, if the sample of extraneous antigen used for comparative titrations contained little of the active material. If at first this 20-fold difference in the titrations cannot be obtained, it might be more profitable to spend time purifying the extraneous antigen sufficiently to attain the desired difference in titrations, rather than to attempt to remove from the purified product what may be a relatively small contamination with extraneous antigen. When the final product, which passes this criterion of purity, is examined chemically, it would be advisable to check that any peculiar chemical attribute is not possessed by the (impure) samples of extraneous antigens.

As an example, the final preparation of factor I of the anthrax toxin produced *in vitro*—described above—was examined by the above procedure. In addition to the line associated with factor I activity, which did not appear to be composite, the final preparation (0.5 %, w/v) formed one faint line against 'spore' antiserum (H 533). Against 'antigen' antiserum (H 25) it (0.5 %, w/v) formed two faint lines. The faint line formed with antiserum H 533 corresponded with one of the two lines formed with antiserum H 25 and both lines were due to extraneous antigens, since they were formed by smaller amounts of relatively inactive fractions discarded during fractionation of factor I. A short purification of these inactive fractions on diethylaminoethyl cellulose columns produced samples of each extraneous antigen; these were used in comparative titrations with the final preparation of factor I on gel diffusion plates against both types of anthrax antiserum. These titrations indicated that the final preparation of factor I was contaminated with not more than 0.5 % of one extraneous serologically-reacting material and not more than 5 % of the other.

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Oxidation of *meso*- α,ϵ -Diaminopimelic Acid by Certain Sporulating Species of Bacteria

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SUMMARY

Investigations were made of the transformations undergone by the stereoisomers of α,ϵ -diaminopimelic acid in suspensions of acetone-dried organisms of two species of sporulating bacteria, *Sporosarcina ureae* and *Bacillus sphaericus*, both of which contain diaminopimelic acid in their spores but not in their vegetative cells. *Meso*-diaminopimelic acid was rapidly decarboxylated by vegetative organisms of both species; it was also utilized by some other unidentified anaerobic reaction. The vegetative organisms also oxidized *meso*-diaminopimelic acid with release of ammonia. L-Lysine was oxidized by *S. ureae*, but not by *B. sphaericus*. Neither LL- nor DD-diaminopimelic acid was attacked by either organism.

Disintegrated spores of *Bacillus sphaericus* did not oxidize *meso*-diaminopimelic acid, but decarboxylated it and also utilized it by the unidentified anaerobic reaction. The decarboxylation, but not the oxidation, of diaminopimelic acid by *Sporosarcina ureae* was greatly stimulated by pyridoxal phosphate; both reactions were inhibited by the same compounds. Study of the oxidation was complicated by the side reactions which occurred with *S. ureae*, but a simpler system was provided by an asporogenous variant of *B. sphaericus* which did not decarboxylate diaminopimelic acid without added pyridoxal phosphate. Only one equivalent of ammonia was produced, a small amount of CO₂ was evolved and two equivalents of oxygen were utilized; no oxidation product was identified. The methods of attacking diaminopimelic acid by these two atypical species are compared and discussed in relation to other species in their respective families.

INTRODUCTION

α,ϵ -Diaminopimelic acid differs from the majority of the common natural amino acids in being confined almost exclusively to bacteria where it occurs mainly in the cell walls (Work & Dewey, 1953; Work, 1957*a*, 1961). However, certain of its stereoisomers undergo enzymic reactions resembling those of other amino acids: thus, in many types of bacteria the *meso*-isomer is decarboxylated to L-lysine (Dewey, Hoare & Work, 1954) and is also racemized to the LL-isomer (Antia, Hoare & Work, 1957); all three isomers can transaminate (Meadow & Work, 1958*a*) while L-amino acid oxidases of *Neurospora* and snake venoms oxidize *meso*- and LL-diaminopimelic acid (Work, 1955). Studies on the decarboxylation of *meso*-diamino-

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pimelic acid by acetone-dried bacteria (Dewey *et al.* 1954) have not so far suggested that many of these preparations can carry out other types of reaction with the amino acid. Thus, the rate of decarboxylation was not usually affected by the presence of oxygen, and the volume of gas evolved, both aerobically and anaerobically, corresponded to that expected from the release of one mole CO_2 /mole *meso*-diaminopimelic acid added. In the two species now used, *Sporosarcina ureae* and *Bacillus sphaericus*, neither of these conditions was found; the rate of CO_2 evolution from *meso*-diaminopimelic acid by acetone-dried organisms was apparently lowered by the presence of air, and the amount of CO_2 produced was less than stoichiometric even under anaerobic conditions.

Certain relationships between bacterial classification and the distribution of diaminopimelic acid and of its decarboxylase and racemase have been established in a few families (Work & Dewey, 1953; Antia *et al.* 1957; Dewey, 1954; Hoare & Work, 1957; Cummins & Harris, 1956*a, b*; Cummins, 1956). As a consequence, the species studied in this communication can be differentiated from other members of their families by their content of diaminopimelic acid and of enzymes attacking it (Table 2). *Sporosarcina ureae* is a spore-forming member of the family Micrococcaceae. Typical organisms in this family show diaminopimelic acid decarboxylase and racemase activities, but do not contain the amino acid itself (Antia *et al.* 1957; Cummins & Harris, 1956*b*); *S. ureae* has an active decarboxylase but no racemase. As described in this paper it has diaminopimelic acid only in its spores and not in its vegetative cells. *Bacillus sphaericus* is classified with the other aerobic spore-forming bacilli, most members of which contain diaminopimelic acid and its racemase but no decarboxylase. No diaminopimelic acid racemase was found by Powell & Strange (1957) in *B. sphaericus*, but there was a very active decarboxylase; diaminopimelic acid itself was not present in vegetative cells, but was present in the spores.

This paper describes the reactions undergone by diaminopimelic acid in suspensions of acetone-dried vegetative cells of these two species of bacteria which are atypical with respect to diaminopimelic acid content and metabolism. The anomalies of aerobic decarboxylation were due to oxidation, which has not been hitherto observed in bacteria.

METHODS

Organisms. The strain of *Sporosarcina ureae* studied originated from the Microbiological Laboratory, Technical High School, Delft. Another strain from the Edinburgh and East of Scotland College of Agriculture was examined briefly. The organism was subcultured weekly in a medium (called hereafter urea medium) containing urea 0.5% (w/v), Lab-Lemco 1% (w/v), peptone (Oxoid) 1% (w/v), adjusted to pH 7.0 with NaOH and filtered. The organism was grown at room temperature (18°) and cultures were stored at 0°. Other media used for this organism were nutrient broth fortified with tryptic digest of casein (0.5 g. nitrogen/100 ml.) called TMB broth, the CCY medium of Gladstone & Fildes (1940), and the meat extract-peptone of Tarr (1933). Preparations of the vegetative form were grown with shaking at 18°; for each 100 ml. of medium, 1 ml. of a 48 hr. culture in the urea medium was used as inoculum after adjusting to a standard optical density. After 24 or 48 hr. growth, the organisms were harvested by centrifugation,

washed twice with 0.9% (w/v) NaCl solution and acetone-dried in the cold. They were stored at -15° .

Bacillus sphaericus was var. *fusiformis* NCTC 7582, and its asporogenous variant; these strains were studied by the late Mrs J. F. Powell and her colleagues (Powell & Strange, 1957; Powell 1958; Powell & Hunter, 1955). The organisms were grown at 37° by Mrs Powell in potato-extract medium enriched with CCY (Meadow & Work, 1958*b*); for vegetative preparations the growth period was 10 hr.; for spore preparations it was 48 hr. The harvested organisms were washed three times with water, and either acetone-dried or freeze-dried and stored at -15° . The spores were disintegrated mechanically in a Mickle disintegrator for 40 min. at 0° (20 mg. dry wt./ml.) in the presence of thiolacetic acid (mM) with octyl alcohol as antifoaming agent.

Other organisms. The strains and growth conditions were described by Antia *et al.* (1957).

Enzymic reactions. Decarboxylation and oxidation were measured in Warburg manometers at 37° . In both cases 0.1 M-phosphate buffer (pH 6.8) was used with 20–40 mg. dried organism suspended in a total volume of 2.5 ml. Unless otherwise stated, the substrates (tipped from side arm) were either *meso*-diaminopimelic acid (2 mg.; final concentrations of 4.2 mM) or L-lysine (15 mM); pyridoxal phosphate, when present, was $10\mu\text{M}$. Decarboxylation was carried out in an atmosphere of N_2 ; when gas evolution had ceased, m-citric acid (0.1 ml.) was tipped in from a second side arm to release CO_2 from solution (Hoare & Work, 1955). Oxidation was carried out in air with 20% KOH (0.2 ml.) and filter paper in the centre well. Control flasks without substrate were included, and were corrected for when calculating the final reaction rates. Carbon dioxide output (Q_{CO_2}) was expressed as $\mu\text{l. CO}_2$ produced/mg./hr. (not corrected for gas retention), and oxygen uptake (Q_{O_2}) as $\mu\text{l. O}_2$ taken up/mg./hr.

The manometric balance experiments were carried out essentially according to the method described under 'direct method for estimation of CO_2 ' (Umbreit, Burris & Stauffer, 1957). Pairs of flasks were set up, aerobically and anaerobically, containing either KOH or water in the centre well; this was done in the presence and in absence of synthetic diaminopimelic acid (8 mg./2.8 ml.). By this means the amounts of CO_2 evolved by oxidation and of O_2 absorbed were calculated. At the same time, the suspensions were incubated in open flasks with identical proportions of diaminopimelic acid, and samples were withdrawn at intervals for estimation of ammonia and diaminopimelic acid.

Examination of reaction mixtures. When not investigated immediately, the final reaction mixtures from the Warburg flasks were frozen rapidly and stored at -15° . Measured amounts of the thawed mixtures were examined as required.

Paper chromatography. The reaction mixtures were usually deproteinized and freed from organisms by treatment with 2 vol. ethanol and centrifugation before paper chromatography without preliminary hydrolysis; under certain circumstances the mixture was used after removal of the organisms by centrifugation only. The equivalent of 0.1 ml. of reaction mixture was examined by two-dimensional chromatography on Whatman no. 4 paper using as solvents aqueous phenol (NH_3 atmosphere) and *n*-butanol (4) + acetic acid (1) + water (5). Alternatively, $33\mu\text{l.}$ was examined for diaminopimelic acid on one-dimensional chromatograms on

no. 1 paper with the solvent methanol (80) + pyridine (10) + 10 N-HCl (2.5) + water (17.5) (Hoare & Work, 1955). Amino acids were revealed by dipping the chromatograms in ninhydrin in acetone (0.1%, w/v) and heating at 102°. When required, hydrolysis of reaction mixtures or of bacteria was carried out with 6 N-HCl for 24 hr.

Estimation of diaminopimelic acid. Ninhydrin in strong acetic acid was used to estimate diaminopimelic acid colorimetrically (Work, 1957*b*). For the balance experiments, synthetic diaminopimelic acid (mixture of *meso*, LL- and DD-isomers) was used, in preference to the *meso* isomer (the only form decarboxylated), in order to decrease the amount of reaction mixture necessary to give a measurable colour, and so to avoid high blank values due to the intracellular amino acids. Two flasks were used, one contained diaminopimelic acid, the other was a control. The mixtures were shaken at 37°, and at intervals samples were deproteinized with an equal volume of acetic acid and centrifuged after coagulation was complete; 0.1 ml. of the supernatant solution was mixed with water (0.4 ml.), acetic acid (0.5 ml.) and ninhydrin reagent (0.5 ml.). Ninhydrin reagent b was used (Work, 1957*b*); it consisted of ninhydrin (A.R. Grade) 250 mg., acetic acid 6 ml., 0.6 M-phosphoric acid 4 ml. The solutions were heated at 100° for 2 min., cooled and diluted with acetic acid (3.5 ml.); the optical density at 440 m μ was read against the mixture from the control flask. A standard curve was constructed by adding known amounts of synthetic diaminopimelic acid to the deproteinized contents of the control flask. The short heating time of 2 min. was used to minimize colour formation from intracellular amino acids and also from lysine formed by decarboxylation of *meso*-diaminopimelic acid. Estimations on other reaction mixtures were carried out by essentially the same method, with slight differences introduced according to conditions.

Estimation of ammonia. The reaction mixture (0.5 ml.) was aerated for 1 hr. at room temperature in the presence of 4 ml. water + 1 ml. saturated K₂CO₃ + 1 drop triamylcitrate. The ammonia carried over was trapped in 15 ml. of 0.04 N-H₂SO₄ and measured colorimetrically after treating 5 ml. of the distillate (diluted with an equal volume of water) with Nessler's reagent (0.5 ml.). The optical density at 450 m μ , read within 2 min. against that of a reagent blank, was compared against that of a standard curve previously constructed with solutions from known amounts of ammonium sulphate treated identically. The ammonia contents of the control suspensions incubated without added substrate were subtracted from those of the test solutions.

Qualitative examination for keto-acids. Keto-acids were examined in the reaction mixtures by a modification of the method of el Hawary & Thompson (1953). Some of the yellow dinitrophenylhydrazones of keto-acids were apparently unstable in the K₂CO₃ solution used to extract them from ethyl acetate, therefore the alkaline extractions were carried out at 0° and the extracts neutralized immediately with cold 3 N-HCl. After re-extraction into ethyl acetate and drying over anhydrous K₂CO₃, the extracts were examined by ascending paper chromatography in *n*-butanol (70) + ethanol (10) + 0.5 N-ammonia (20).

Examination for dipicolinic acid. This was carried out as suggested by Powell & Strange (1957); after deproteinization of the reaction mixtures with perchloric acid and dilution with 50 volumes of buffer (pH 7.3) the light absorption between 240 and 280 m μ was measured in the Beckmann spectrophotometer.

RESULTS

Sporosarcina ureae

Diaminopimelic acid in *Sporosarcina ureae*. Both strains of *S. ureae*, examined shortly after their arrival in the laboratory, yielded organisms which contained small amounts of diaminopimelic acid: subsequently, no diaminopimelic acid was found in whole or fractionated organisms grown in liquid culture. One culture, grown on solid medium, contained a trace of diaminopimelic acid and was found to consist of a mixture of vegetative organisms and spores. All further attempts in our laboratory to produce sporulation of either strain failed, but later Powell & Hunter (private communication) succeeded once in obtaining spores from the Edinburgh strain. These spores contained *meso*-diaminopimelic acid, the vegetative organisms had none.

Table 1. *Degradation of meso-diaminopimelic acid and L-lysine by acetone-dried Sporosarcina ureae*

Experiments were carried out in Warburg manometers with 40 mg. dried organism in total volume of 2.5 ml. 0.1 M-phosphate buffer (pH 6.8). Anaerobic experiments in nitrogen, aerobic in air with KOH in centre cup. Pyridoxal phosphate when present was 10 μ M. Other conditions as in Methods.

Culture				Manometric experiment								
				meso-Diaminopimelic acid								
Expt. no.	Growth conditions		Yield (g. dry wt./l.)	Pyridoxal phosphate	Anaerobic		Aerobic				L- Lysine Aerobic	
	Medium	Time (hr.)			Q _{CO₂}	V* _{CO₂}	Lag (min.)	Q _{O₂}	V* _{O₂}	V* _{H₃}	Q _{O₂}	V* _{O₂}
1	Urea	24	1.46	+	9.3	92	3.0	87
				0	3.4	3.0	87
1	Urea	48	1.45	+	8.7	89	15	3.8	.	.	5.5	87
				0	4.5	5.5	94
2	Urea	48	1.10	+	6.9	77	16	2.3	106	.	.	.
				0	5.4	56	16	2.0	117	38	.	.
3	CCY	24	1.01	+	8.8	87	30	1.3	>142	.	0.6	.
3	CCY	48	1.60	+	8.6	94	19	1.6	>165	36	0.6	.
4	CCY	48	1.92	+	9.3	78	18	2.6	154	50	3.7	.
				0	7.4	63	20	2.5	157	50	.	.
5	TMB broth	48	1.48	+	7.7	85	37	0.8	32	21	.	.
				0	3.1	40	15	0.3
6	Tarr spore broth	48	0.73	+	7.0	86	27	0.7
				0	4.3	54	24	0.7

* Volume of reactants used or produced expressed as % of theoretical, based on 1 equivalent/mole amino acid present.

$Q_{CO_2} = \mu$ l. CO_2 evolved/hr./mg. dry wt. $Q_{O_2} = \mu$ l. oxygen absorbed/hr./mg. dry wt.

Anaerobic transformations of diaminopimelic acid. Acetone-dried organisms from both strains of *Sporosarcina ureae* grown on urea medium decarboxylated *meso*-diaminopimelic acid; the rate with the Delft strain was about double that with the Edinburgh strain. Thereafter all experiments were carried out with the Delft strain. The rate of decarboxylation did not vary greatly with change in growth conditions

(Table 1); the highest activities ($Q_{\text{CO}_2} = 9.3$) were observed with organisms grown either on urea medium or on CCY medium. Omission of pyridoxal phosphate from the Warburg flasks lowered the decarboxylation rate. There was no decarboxylation reaction with LL-diaminopimelic acid, indicating the absence of diaminopimelic racemase (Antia *et al.* 1957).

The identifiable products of reaction were CO_2 and lysine. The organisms contained no lysine decarboxylase; an equimolar volume of CO_2 should therefore have been liberated from *meso*-diaminopimelic acid, but this was seldom the case (Table 1). Gas evolution stopped at 80–90% of the theoretical value when pyridoxal phosphate was added, and at 40–80% of theoretical without added pyridoxal phosphate. In spite of this, only 1–5% of the original *meso*-diaminopimelic acid remained in the final reaction mixtures. Additional *meso*-diaminopimelic acid caused resumption of gas evolution, even in the absence of added pyridoxal phosphate. These results suggested that, in addition to decarboxylation, *meso*-diaminopimelic acid was undergoing some anaerobic reaction which was not stimulated by pyridoxal phosphate. Paper chromatography showed that the diaminopimelic acid unaccounted for had not been incorporated as a peptide or other form liberated by hydrolysis, either in the soluble or the insoluble portions of the reaction mixtures.

Aerobic breakdown of diaminopimelic acid. When the decarboxylation of *meso*-diaminopimelic acid by acetone-dried *Sporosarcina ureae* was carried out in air, the gas output was slower and lower than under anaerobic conditions (Fig. 1, curves A and B). In spite of this diminished CO_2 production, both diaminopimelic acid and lysine disappeared completely from the aerobic reaction mixture, while no other amino acids were produced. When the CO_2 evolved was absorbed by KOH, a steady uptake of gas occurred after a short lag entailing a slight gas output (curve D). A gas uptake also occurred with L-lysine (curve E and Table 1), but there was no lag. No gas uptake occurred with LL-diaminopimelic acid (curve C), DD-diaminopimelic acid, D-lysine, L-alanine, L-glutamic acid or glucose. It was evident that oxidative reactions had occurred with both *meso*-diaminopimelic acid and L-lysine. In the case of diaminopimelic acid, simultaneous oxidation and decarboxylation could result in curves such as B and D; in curve B where no KOH was present, oxygen uptake would produce a decrease in overall gas evolution. The preliminary lag in curve D can be attributed to the inability of KOH to absorb all the CO_2 released by decarboxylation in the first few minutes; when KOH was present during anaerobic decarboxylation of *meso*-diaminopimelic acid, there was also a considerable delay in the absorption of CO_2 , but finally there was no over-all change in volume. There is, however, a possibility that only lysine was oxidized by the preparation, and that the lag in oxygen uptake with diaminopimelic acid was due to preliminary decarboxylation to lysine. The reactions were therefore studied further. Fresh suspensions or broken-cell suspensions could not be used because of their high endogenous respiration rate; in acetone-dried organisms this was not unduly high ($Q_{\text{O}_2} = 1.1$ – 1.8 for organisms grown on CCY medium, 0.5 – 0.8 for other organisms), and was allowed for in calculating oxidation rates of the various substrates.

The rate of oxidation of *meso*-diaminopimelic acid was raised only slightly by the addition of pyridoxal phosphate (Table 1), that of lysine was unaffected. The rates

and extents of oxygen consumption varied with the conditions of growth, particularly in the case of L-lysine, but they also varied from one preparation to another of organisms grown under the same conditions. The total oxygen consumed was usually between 80 and 100 % of the theoretical value calculated for one atom of oxygen taken up by one molecule of either amino acid; but in some cases, particularly when the organisms were grown on CCY medium, the oxygen consumed

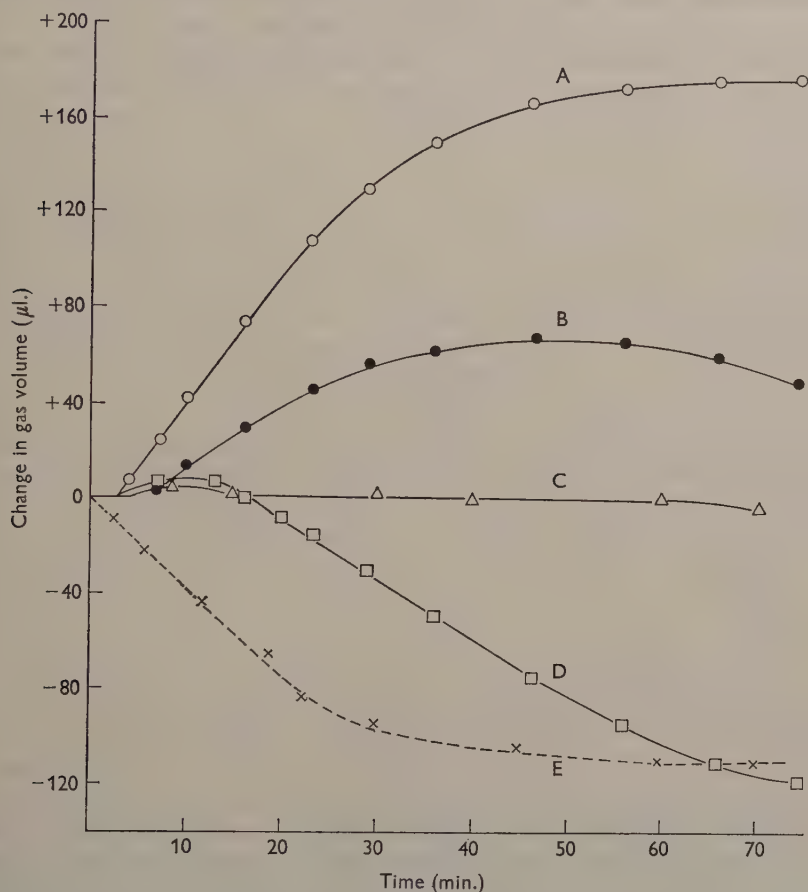


Fig. 1. Behaviour of diaminopimelic acid and lysine in the Warburg apparatus in presence of acetone-dried *Sporosarcina ureae*. Each flask contained 40 mg. dried organism (grown 48 hr. in urea medium) in 0.1 M-phosphate buffer (pH 6.8); total volume 2.5 ml.; present $10\mu\text{M}$ pyridoxal phosphate. Substrates tipped from side arms were as follows: curves A, B and D, *meso*-diaminopimelic acid (4.2 mM); curve C, L-diaminopimelic acid; curve E, L-lysine (5.5 mM). Curve A, atmosphere N_2 ; curve B, atmosphere air, no KOH present; curves C, D, E, atmosphere air, KOH present. Changes in volume were corrected for those in control flasks without substrate.

by *meso*-diaminopimelic acid exceeded this value. There was no constant relation between the rates of oxidation of L-lysine and the decarboxylation of *meso*-diaminopimelic acid by different batches of organisms. The minimum concentrations of *meso*-diaminopimelic acid and L-lysine required to produce maximum oxidation rates were 2 and 15 mM, respectively.

The effects of inhibitors on the decarboxylation and oxidation of *meso*-diaminopimelic acid were investigated in the hope that decarboxylation might be inhibited specifically. Compounds which bind thiol or carbonyl groups inhibit diaminopimelic acid decarboxylase from *Aerobacter aerogenes* (Dewey *et al.* 1954; Hoare, 1956). Both oxidation and decarboxylation by *Sporosarcina ureae* were inhibited to the same extent by any one of these inhibitors.

Ammonia was always produced by these aerobic reactions, whereas none was produced anaerobically. Ammonia could only be estimated reliably in the products from *meso*-diaminopimelic acid oxidation; in the case of L-lysine, duplicate estimations did not agree, suggesting that a reaction product was unstable under the

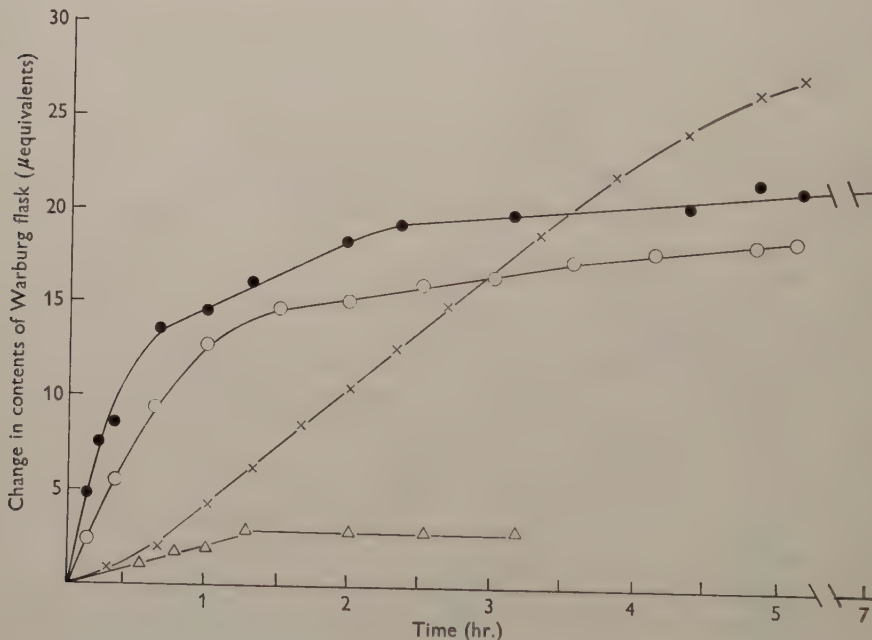


Fig. 2. Balance experiment showing aerobic decomposition of *meso*-diaminopimelic acid by acetone-dried *Sporosarcina ureae*. Final reaction mixture contained dried organism (grown CCY medium for 48 hr.) 1.6 mg./ml. in 0.1 M-phosphate buffer (pH 6.8) containing $10\mu\text{M}$ pyridoxal phosphate. Pairs of Warburg flasks were used containing total volumes of 2.8 ml.: in one flask of each pair there was 8 mg. ($42\mu\text{mole}$) of synthetic diaminopimelic acid, the other contained no substrate. In two pairs the reaction was carried out aerobically; KOH (0.2 ml. of 20%) was in the centre well of only one pair. In a third pair of flasks KOH was present but the atmosphere was N_2 . The same suspension was also incubated in two open flasks, with and without diaminopimelic acid, at the same concentration as in the Warburg flasks; samples were withdrawn at intervals for estimation of ammonia and diaminopimelic acid. Changes in gas volumes were calculated as in Umbreit *et al.* (1957). Results are expressed as change in amounts of reactants in a Warburg flask. \times — \times = oxygen taken up; O — O = CO_2 evolved (not corrected for retention in solution); \bullet — \bullet = diaminopimelic acid disappearing; Δ — Δ = ammonia formed.

alkaline conditions of the ammonia distillation. The amount of ammonia produced during oxidation of diaminopimelic acid bore no constant relation to the oxygen uptake or to the amount of diaminopimelic acid utilized, but the molar ratio of

oxygen or diaminopimelic acid consumed to ammonia produced was always greater than unity (Table 1).

When the reaction products from diaminopimelic acid oxidation were examined by chromatography in methanol+water+pyridine+HCl and revealed with ninhydrin, a yellow spot having a pink fluorescence in ultraviolet radiation was often observed just behind the solvent front. The substance giving this spot has not been identified; it was not dipicolinic acid or piperidine-2:6-dicarboxylic acid, but it might have been a keto-acid, as suggested by the colour and fluorescence of its ninhydrin reaction product on paper (Rabson & Tolbert, 1958). Examination of the keto-acid dinitrophenyl hydrazones in the reaction mixtures showed very small amounts of oxoglutaric and pyruvic acids and also of an unidentified keto-acid with $R(\text{pyruvate}) = 0.7$; these were insufficient in amount to account for the diaminopimelic acid used.

Balance experiments were carried out, as described in methods, with synthetic diaminopimelic acid. One such experiment is illustrated in Fig. 2. During the first 40 min., in which rapid decarboxylation took place, diaminopimelic acid disappeared rapidly; after this it was utilized more slowly, finally reaching a constant value representing 50% of the original amount present (this is the probable proportion of LL- and DD-isomers in a synthetic mixture). A steady uptake of oxygen continued even after utilization of diaminopimelic acid had stopped; it had not appreciably slowed by the end of the experiment (5 hr.), when 1.25 atom of oxygen had been used per mole of diaminopimelic acid consumed. This high oxygen consumption, typical of organisms grown on CCY medium, showed that in these cells, at any rate, the oxygen uptake was due in part to a secondary reaction. Ammonia production had only reached $2.6 \mu\text{mole}$ at 3 hr. when $20 \mu\text{mole}$ diaminopimelic acid had disappeared.

Degradation of diaminopimelic acid by dried cells of other species of bacteria

In the family Micrococcaceae, the marked stimulation by pyridoxal phosphate of anaerobic decarboxylation of *meso*-diaminopimelic acid was not peculiar to *Sporosarcina ureae*, although the low output of CO_2 was more specific. For example, with *Sarcina lutea*, although the decarboxylation rate was doubled by added pyridoxal phosphate, the CO_2 output was 97% of theoretical even in the absence of added pyridoxal phosphate. Aerobic experiments were difficult to carry out on most Micrococcaceae, because of their high endogenous respiration, even after acetone-drying. With acetone-dried *Sarcina lutea*, this rate was not altered by synthetic diaminopimelic acid or L-lysine; *Staphylococcus citreus* had $Q_{\text{CO}_2} = 8.0$ with no added substrate or with L-lysine, and in the presence of *meso*-diaminopimelic acid $Q_{\text{CO}_2} = 8.3$.

The majority of Bacillaceae do not decarboxylate *meso*-diaminopimelic acid (Antia *et al.* 1957); the known exceptions being *Clostridium tetani* and *Bacillus sphaericus*. *C. tetani* decarboxylated *meso*-diaminopimelic acid faster under anaerobic conditions ($Q_{\text{CO}_2}^{\text{N}_2} = 3.0$) than in presence of air ($Q_{\text{CO}_2}^{\text{air}} = 1.4$). Even anaerobically, the gas output stopped after only 50% of the theoretical amount of CO_2 had been evolved. This suggests that *C. tetani* utilized diaminopimelic acid by the unidentified anaerobic reaction and by oxidation, but no further work was done with this organism.

Bacillus sphaericus. The metabolism of diaminopimelic acid by *B. sphaericus* has been studied in detail, and is described here and elsewhere (Powell & Strange, 1957; Meadow & Work, 1958*b*). Diaminopimelic acid decarboxylase activity was very high in freeze-dried and acetone-dried vegetative *B. sphaericus*, and was greatly stimulated by pyridoxal phosphate, rates of the order of $Q_{CO_2}^{N_2} = 30$ being found under optimal conditions. Anaerobic decarboxylation of *meso*-diaminopimelic acid evolved suboptimal amounts of CO_2 , especially in the absence of pyridoxal phosphate when only about 60% of the theoretical volume was produced (Fig. 3A, curve 1).

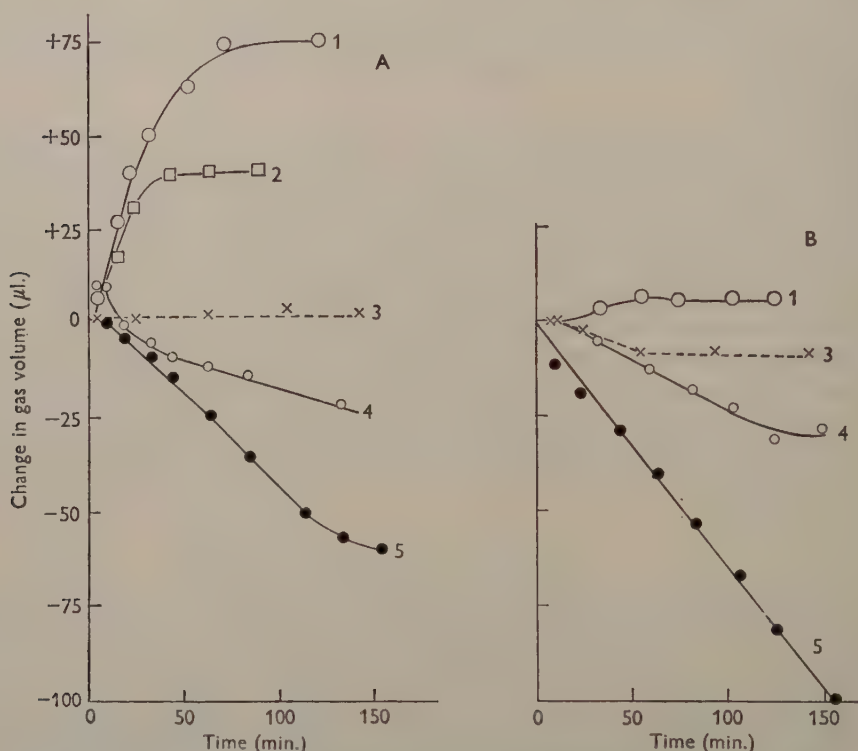


Fig. 3. Behaviour of *meso*-diaminopimelic acid in Warburg apparatus in presence of acetone-dried *Bacillus sphaericus*. Each flask contained a suspension of dried vegetative organisms (20 mg.) in 0.1 M-phosphate buffer (pH 6.8); final volume 2.5 ml. A; *B. sphaericus*, var. *fusiformis* NCTC 7582, normal strain. B; Asporogenous variant of this strain. All solid curves represent substrate *meso*-diaminopimelic acid (2 mg.), \times --- \times = substrate L-lysine (6 mg.). Curve 1, decarboxylation, atmosphere N_2 , no KOH; curve 2, atmosphere air, no KOH; curves 3 and 5, oxidation, atmosphere air, KOH present; curves 1, 2, 3 and 5, no pyridoxal phosphate added. Curve 4, oxidation, atmosphere air, KOH present, pyridoxal phosphate (10 μM) added.

The rate of gas evolution was almost unaffected by the presence of air, but in this case the volume of gas evolved was lower (Fig. 3A, curve 2). In all experiments, 95–100% of the added diaminopimelic acid disappeared; with the exception of lysine no other amino acids were formed. No evidence for binding of diaminopimelic acid was obtained. Aerobic experiments in the presence of KOH resulted in slow gas uptakes with *meso*-diaminopimelic acid (Fig. 3A, curves 4 and 5). These

results resembled those found in acetone-dried *Sporosarcina ureae*, and suggested that in *B. sphaericus* meso-diaminopimelic acid was subjected to both the unidentified anaerobic reaction and oxidation. However, owing to very rapid decarboxylation, even in the absence of pyridoxal phosphate, the direct observation of oxidation of diaminopimelic acid was even more difficult than in the case of *S. ureae*. On the other hand, *B. sphaericus* did not oxidize L-lysine (Fig. 3A, curve 3), so that any observed oxygen uptake could be attributed directly to diaminopimelic acid even if decarboxylation were occurring at the same time. The oxygen uptakes in the absence of substrate were negligible with acetone-dried organisms. Disintegrated suspensions of fresh spores of *B. sphaericus* showed lower values for diaminopimelic acid decarboxylase ($Q_{CO_2} = 4.1$) than did the vegetative organisms. Neither the rate nor the extent of decarboxylation by spores was affected by the presence of oxygen or of pyridoxal phosphate; the volume of CO_2 evolved was always about 60 % if theoretical. Diaminopimelic acid was evidently not oxidized by spores, but the decarboxylase was active and fully saturated with pyridoxal phosphate; the unidentified anaerobic reaction also occurred.

The asporogenous variant of *Bacillus sphaericus* is known to have no diaminopimelic acid decarboxylase activity (even when acetone-dried) unless pyridoxal phosphate is added (Meadow & Work, 1958*b*). In the absence of this coenzyme, the unknown anaerobic reaction evidently also does not occur, since meso-diaminopimelic acid was unchanged in concentration after anaerobic incubation for 5 hr. with acetone-dried cell suspensions (Work, 1957*b*). However, meso-diaminopimelic acid was oxidized (Q_{O_2} between 2.0 and 3.0), whereas L-lysine and the other isomers of diaminopimelic acid were not attacked (Fig. 3B). The gas uptake with meso-diaminopimelic acid started immediately, without a preliminary lag (Fig. 3B, curve 5). Since pyridoxal phosphate had already been found to have little effect on the oxidation of diaminopimelic acid by *Sporosarcina ureae*, it was decided that the asporogenous variant of *B. sphaericus* would be a good material for a study of the reaction.

The pH optimum for the oxidation of meso-diaminopimelic acid by a suspension of acetone-dried *Bacillus sphaericus* (asporogenous) lay between 6.8 and 7.4, outside this range a rapid fall in reaction rate was noted (at pH 6.0 and 7.8 the rates were only 14 % of the maximum).

Balance experiments showed that the reaction with asporogenous *Bacillus sphaericus* was more straightforward than in the case of *Sporosarcina ureae*, as the amounts of ammonia produced and oxygen taken up were proportional to the diaminopimelic acid utilized. For example, in an oxidation, not carried to completion, of 20.6 μ mole synthetic diaminopimelic acid by 30 mg. acetone powder, in 165 min. 5.5 μ mole ammonia were produced, 5.8 μ mole of diaminopimelic acid disappeared and 13 μ equivalents of oxygen were taken up. In another experiment, in 45 min., 2 μ equivalents of oxygen were used by 20 mg. of acetone powder and 0.6 μ mole CO_2 produced; during the same period, only 0.2 μ mole CO_2 was evolved anaerobically. No dipicolinic acid was formed during the aerobic reaction. There were trace amounts of the keto acid with a dinitrophenyl hydrazone having a mobility of R (pyruvate) = 0.7 on paper chromatograms.

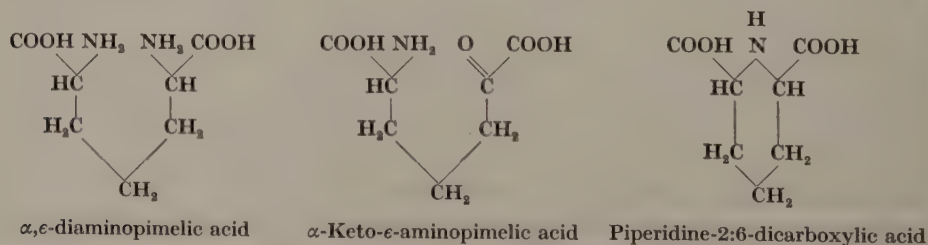
These experiments indicate that the reaction involved in meso-diaminopimelic acid oxidation by the asporogenous strain of *Bacillus sphaericus* is probably oxidative removal of one amino group, followed perhaps by oxidative splitting of the

carbon chain or by decarboxylation. Another batch of asporogenous cells was grown, but the preparation showed very weak oxidase activity, although it still retained an active apodecarboxylase. Since this organism has been found to change very much in successive subculture (Meadow & Work, 1958*b*), it was not considered to be suitable for further study of diaminopimelic acid oxidation. The work has been temporarily abandoned.

DISCUSSION

The experiments described showed that *meso*-diaminopimelic acid, but not the LL- or DD-isomers, is oxidized by acetone-dried vegetative organisms of two species of sporulating bacteria which are atypical in their contents of diaminopimelic acid and in their methods of utilizing this substance (summarized in Table 2). This stereochemical specificity of the oxidation, easily demonstrable owing to the unusual absence of diaminopimelic acid racemase from the organisms in question, distinguishes the reaction from the less stereospecific oxidations which both the LL- and *meso*-isomers undergo with L-amino acid oxidases from *Neurospora* or snake venoms (Work, 1955). The reaction probably involves a deamination, but whether this is the primary reaction is not yet known. For example, a transamination, catalysed by small amounts of keto acids found in the organisms, could remove one amino group, and the resulting α -keto- ϵ -aminopimelic acid might then be oxidized with or without loss of its amino group. The stereochemical specificity of the oxidation does not support this theory, since both LL- and *meso*-diaminopimelic acid are transaminated by the organisms in question (see Meadow & Work, 1958*a*, for transamination by *Bacillus sphaericus*). The virtual independence of added pyridoxal phosphate of the oxidative reaction by *Sporosarcina ureae* also suggests that it was not connected with transamination, which requires high concentrations ($40\mu\text{M}$) of this coenzyme in acetone-dried organisms. The neutral pH value at which the oxidation occurs also does not favour transamination.

The failure to find significant amounts of keto acids in the reaction mixtures is not contra-indicative of an oxidative deamination, since α -keto- ϵ -aminopimelic acid would be expected to cyclize spontaneously by condensation of the α -keto and ϵ -amino groups, as in the case of α -keto acid resulting from oxidation of lysine (Meister, 1954). α -Keto- ϵ -aminopimelic acid is known to be an intermediate in the biosynthesis of LL-diaminopimelic acid in *Escherichia coli*, but in this case it is present as the N-succinyl derivative and is thereby protected against cyclization (Gilvarg, 1960).



Neither piperidine-2:6-dicarboxylic acid, a reduced product of ring closure, nor dipicolinic acid, the fully unsaturated derivative which occurs in bacterial spores (Powell, 1953), was identified among the reaction products.

Although *Sporosarcina ureae* and *Bacillus sphaericus* are organisms belonging to different families, their methods of utilizing diaminopimelic acid (Table 2) are sufficiently similar to enable them to be compared and to be differentiated from other organisms in their respective families. *Clostridium tetani* may be a similar exception: it is the only other member of the Bacillaceae known to decarboxylate diaminopimelic acid and sometimes to lack it in its vegetative cells.

Table 2. Comparison of certain characteristics in typical and atypical members of families micrococcaceae and bacillaceae

Characteristic	Micrococcaceae		Bacillaceae		
	Typical species	<i>Sporosarcina ureae</i>	Typical species	<i>Bacillus sphaericus</i>	<i>Clostridium tetani</i>
DAP* in vegetative cells	—	—	+	—	Not invariably
DAP in spores	No spores	+	+	+	?
DAP decarboxylase†	+	+	—	+	+
Alternative anaerobic reaction†	—	+	?	+	+
DAP racemase†	+	—	+	—	Slight
DAP oxidation†	—	+	—	+	Possible

* Diaminopimelic acid.

† Reactions investigated in acetone-dried vegetative organisms.

Diaminopimelic acid is an important constituent of the mucopeptide of the cell walls of many Gram-positive bacteria such as Bacillaceae; whenever it is absent, as in the Micrococcaceae, it is replaced in the wall by lysine (Cummins & Harris, 1956b). In the case of *Bacillus sphaericus*, Powell & Strange (1957) found that the soluble mucopeptides obtained by enzymic degradation of walls of vegetative organisms and spores had similar compositions except for the presence of lysine in the former and diaminopimelic acid in the latter. They also showed variations in the cellular activities of diaminopimelic acid decarboxylase throughout the growth and sporulation cycle, finding a marked decrease in activity in ageing cultures coincident with the appearance of spores and soluble mucopeptides containing diaminopimelic acid. We found that, in contrast to the vegetative cells, spores of this organism did not oxidize diaminopimelic acid.

It is possible that diaminopimelic acid is synthesized in vegetative cells of both *Bacillus sphaericus* and *Sporosarcina ureae*, but that before it can be inserted into the walls it is degraded by the three types of enzymes present in these cells. It is notable that the activities of decarboxylase found in normal dried vegetative cells of *B. sphaericus* and of *S. ureae*, are respectively, 10 and 3 times higher than those found in most other bacteria (Antia *et al.* 1957); however, considerably lower quantities were present in the asporogenous variant of *B. sphaericus*, which also has lysine in its cell wall. Nothing is known of the metabolic determinant which causes the change from a vegetative cell containing lysine in its wall mucopeptides to a spore containing diaminopimelic acid.

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Interaction of an Endotoxin with Cationic Macromolecules

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SUMMARY

The interaction of the anionic lipopolysaccharide complex isolated from a Gram-negative bacterium (*Serratia marcescens*) was studied in aqueous and saline solution with several cationic macromolecules, with the object of selectively inhibiting certain of the biological activities of this polysaccharide endotoxin. Incubation with lysozyme decreased the pyrogenic activity, while the tumour-damaging ability of this polysaccharide remained high. This was in contrast to interaction with RNase and with polymyxin B, in which cases the tumour-damaging activity was decreased while the pyrogenic activity was not affected. Ultracentrifuge experiments indicated that both the tumour-necrotic and especially the fever-producing fractions of this bacterial polysaccharide preparation are of much higher molecular weight ($> 10S$) than the major component ($3.4S$), which is either inactive or of low activity. The highest molecular weight fever-producing components appeared to be broken down in an enzymic type of process by the lysozyme.

INTRODUCTION

The endotoxins from Gram-negative bacteria are high molecular weight lipopolysaccharide complexes which exhibit a whole array of biologic properties when injected into animals in microgram quantities: they are toxic and antigenic, produce fever, cause localized haemorrhage and necrosis in prepared skin sites and in tumours, etc. (cf. Burrows, 1951; Thomas, 1954). There is no *a priori* reason why all of these different biological properties should be caused by the same morphological or physico-chemical entity. For example, it might be that those components or molecular sites in the complex which cause the tumour-damaging activity are not the ones which cause the fever. However, prior attempts to separate the physico-chemical entities responsible for these different biological activities have all resulted in failure and no fractionation or other method has been found to be effective (Rathgeb & Sylven, 1954; Malmgren, 1954; Creech, Koehler, Havas, Peck & Andre, 1954). The general opinion developed that the biological activities are just different manifestations of host reactions to the same physico-chemical entity. When hydrolysis or some other means of splitting the endotoxic lipopolysaccharide complex was tried it led to a total loss of the activities (Rathgeb & Sylven, 1954; Ikawa, Koepfli, Mudd & Niemann, 1952, 1954; Merler, Perrault, Saroff & Mora, to be published). Only when a certain macromolecular size and complexity was maintained, after gentler treatment, were the biological activities retained (Fromme, Luderitz, Nowotny & Westphal 1958; Westphal *et al.* 1958; Ribi *et al.* 1960).

It occurred to us that selective or preferential macromolecular interaction with different components, or sites, of the bacterial polysaccharide preparations might

separate some of these biological properties, while maintaining the necessary macromolecular size.

We reported previously on the blocking of enzyme activity and of toxicity through interaction of oppositely charged macromolecules (Mora & Young, 1958; Mora, Young & Shear, 1959). This approach has been extended to the study of the interaction of a bacterial lipopolysaccharide preparation from *Serratia marcescens* of negative net charge near neutral pH, with positive (cationic) macromolecules. The cationic macromolecules used were polymyxin B, ribonuclease, lysozyme and protamine. Changes in pyrogenic potency and tumour damaging activity were then examined. There was previous evidence that cationic macromolecules affect some of the biological properties of endotoxin preparations (Fisher, 1959; Neter *et al.* 1958). We also studied the possibility of reversal of some of these changes of pyrogenic and tumour damaging activities, when a strong anionic synthetic polysaccharide preparation (polyglucose sulphate) was also added. This was expected to break up the interaction of the macromolecules by preferentially forming a complex with the cationic macromolecules and to restore the biological activity of the anionic bacterial polysaccharide. We found that interaction with lysozyme was qualitatively different from interaction with the other cationic macromolecules; there also was indication of a simultaneous enzymic action.

METHODS

Materials

The endotoxic polysaccharide complex from *Serratia marcescens* (referred to from now on as 'bacterial polysaccharide') was preparation P-25 of A. Perrault, obtained by a modification of the method of Shear & Turner (1943). Fractionation and chemical experiments (Rathgeb & Sylven, 1954*a, b*), electrophoretic and ultracentrifuge studies (Malmgren, 1954) have been carried out previously on this preparation, as well as extensive biological studies.

The cationic macromolecules used were: polymyxin B (Pfizer no. 5 × 070), crystalline ribonuclease (Armour no. 381-059), protamine sulphate (Nutritional Biochemicals Corp. no. 9193), and lysozyme. Lysozyme was used either as obtained commercially (Nutritional Biochemicals Corp. nos. 1417 and 7545) which gave a neutral solution or, when so specified, in a form which we will designate 'basic lysozyme'. This 'basic lysozyme' was obtained from the commercial preparation by bringing to pH 11.4 with dilute NaOH in an aqueous solution and then dialysing this solution in heat-treated cellophan and freeze-drying the residue; this 'basic lysozyme' upon re-solution in water gave a pH of 9.

The anionic polysaccharide derivative was a sulphated polyglucose prepared from a chemically synthesized polyglucose (number average mol. wt. about 20,000) by treatment by chlorosulphonic acid (Prep. H; Wood & Mora, 1958). The polyglucose sulphate contained three sulphate groups per anhydroglucose unit and had intrinsic viscosity (η) = 0.04. The sodium salt or the acid form was used.

Interaction

Interaction of the cationic macromolecules with the bacterial polysaccharide was achieved by mixing dilute aqueous solutions followed by incubation for different

lengths of time. A representative example was as follows: 100 μg . bacterial polysaccharide plus the appropriate amount of basic protein was incubated in 5 ml. pyrogen-free water at 37° for 16 hr. The solution was then diluted with pyrogen-free water for assay of tumour-damaging potency. In the reversal experiments, a concentrated aqueous solution of polyglucose sulphate was added to the incubated aqueous mixture of bacterial polysaccharide and basic protein, further incubated for 1 hr. at 37° , diluted with water and assayed. In the pyrogenic test the final diluent was 0.9% NaCl solution.

Bioassays

Tumour-damaging potency was measured in mice bearing 6-day intramuscular implants of Sarcoma 37, using a method which, with minor variations (Shear, Perrault & Adams, 1943; Landy & Shear, 1957) has been routinely employed in this Laboratory for many years. A few micrograms of the bacterial polysaccharide usually produced, within 24 hr. after intraperitoneal injection, haemorrhage and necrosis in the tumours. In the untreated controls the tumours showed no haemorrhage. Affected tumours were those which showed extensive, freshly induced haemorrhage. A minimum of ten mice was used for each dose level. The minimum effective dose (ED50; the dose which produced extensive damage in tumours of half of the mice) was 5 μg .; this value was reproducible within about $\pm 50\%$.

Pyrogenic activity was measured in rabbits by observing the elevation in rectal temperature under conditions previously described (Landy & Shear, 1957). Lysozyme (16 mg.) was mixed with 20 μg . bacterial polysaccharide, and in the reversal experiment, in addition, with 24 mg. polyglucose sodium sulphate in 1 ml. solution of pyrogen-free water. These aqueous mixtures were incubated for different periods of time at 37° . Thirty minutes before the injection, these concentrated mixtures were diluted with pyrogen-free 0.9% NaCl solution, and 1 ml. samples were injected into the ear vein of the rabbit. The temperature change was then recorded. Four rabbits were used for each dose level. The minimum pyrogenic dose (MPD) of the bacterial polysaccharide was 0.006 μg . ($\pm 50\%$); this produced at least 1°F . fever.

The enzymic potency of lysozyme was determined by measuring the decrease of optical density during the lysis of a suspension of the ultraviolet-treated *Micrococcus lysodeicticus* (Skarnes & Watson, 1955). The lysis was complete in 30 min. The assay provided a method with an error of about 5%.

Chemical methods

The concentration of bacterial polysaccharide was determined by the anthrone procedure, comparing the absorbency at 620 $m\mu$ with that of solutions of known concentration.

Lysozyme or ribonuclease concentrations, in interaction complexes with the bacterial polysaccharide, were estimated after adding salt to bring the mixtures to 0.9% NaCl concentration (which was sufficient to dissolve the precipitates) and then measuring the absorbency of the clear solutions at 280 $m\mu$ and calculating the protein concentration from calibration data. The absorbency due to the bacterial polysaccharide was below the average experimental error (about 5%), and was not used as a correction factor.

Dialysis experiments were carried out in du Pont 400 'gel' cellophan against running distilled water for 64 hr.

Ultracentrifuge experiments

All analytical centrifugation was performed with the Model E Spinco ultracentrifuge using schlieren optics. In the ultracentrifuge experiments the bacterial polysaccharide concentration was 1% in 0.154 M-sodium chloride at 25°. Sedimentation constants are expressed in Svedberg units $S = S_{20w}^{\circ} \times 10^{-13}$ cm./sec. after correction to water at 20° and using the value of 0.6095 for partial specific volume of the bacterial polysaccharide. The moving platform cell and method of Yphantis & Waugh (1956*a, b*) was used in the partition cell analysis experiments.

In the partition cell analysis the centrifugation was at top speed (59,780 rev./min.), and the acceleration and deceleration of the rotor was carefully recorded. After centrifugation the supernatant (upper half), subnatant (lower half of the solution which remained below the partition), rinse of the lower half of the cell, and also an uncentrifuged control solution, were all bioassayed for tumour-damaging activity. Serial dilutions were carried out until the tumour-damaging activity showed a linear decrease with dilution, and the ED50 was determined. The amount of the original tumour-damaging activity present in the particular portion of the cell (upper half, lower half, lower rinse) was then estimated from such data. The pyrogenic activity of these solutions was then similarly determined. In the uncentrifuged control solution the tumour-damaging activity and the pyrogenic activity was present in the expected ratio (ED50 (tumour) 5 µg./mouse, MPD (fever) 0.006 µg./rabbit). From bioassays of the supernatant the sedimentation constant was calculated for both the tumour-damaging and for the fever-producing material. The partition cell analysis essentially followed the method suggested by Yphantis & Waugh (1956*b*) and the calculations took into account the integrated angular velocity during acceleration and deceleration as well as during top speed centrifugation (cf. equations 1-4, Yphantis & Waugh, 1956*a*). The calculated value for the sedimentation constant of the biologically active material was found to vary $\pm 0.5S$ if $\pm 100\%$ error was assumed in the bioassays.

RESULTS

Biological and chemical evidence for interaction

The bacterial polysaccharide at 10-15 µg./mouse caused severe damage in the tumours of most of the mice (Table 1). The same amount of bacterial polysaccharide affected only a small percentage of the tumours after incubation for 30 min. with ribonuclease, polymyxin B, or protamine. Similar incubation with lysozyme, however, led to an opposite result: the tumour-damaging effect of the bacterial polysaccharide increased. The data presented in Table 1 are representative of many such experiments, and also illustrate the reproducibility of the tumour assay.

When polyglucose sulphate was added to the solution containing the bacterial polysaccharide and the polymyxin or RNase, and the incubation was continued for another $\frac{1}{2}$ hr., the decrease in tumour-damaging activity was reversed, and the bacterial polysaccharide essentially recovered its original tumour-damaging activity.

In the case of the solution with lysozyme, the increase in tumour-damaging activity was reversed after further incubation with polyglucose sulphate, and the activity decreased below that of the control.

Table 1. *Tumour-damaging effect of Serratia marcescens polysaccharide and the effect of incubation with cationic macromolecules and polyglucose sulphate*

Bacterial polysaccharide before incubation			After incubation with					
			Cationic macromolecule			Cationic macromolecule and then with polyglucose sulphate		
			Damaged tumours			Damaged tumours		
mg.	no.	%	mg.	no.	%	mg.	no.	%
0.015	17/20	85	1 ribonuclease	2/19	10	0.75	11/20	55
0.015	17/20	85	1 ribonuclease	4/19	21	0.5	19/20	95
0.015	37/40	92	1 ribonuclease	12/39	31	0.5	20/20	100
0.015	17/20	85	0.5 ribonuclease	6/20	30	0.5	19/20	95
0.010	17/20	85	0.25 ribonuclease	2/15	13	0.5	15/15	100
0.015	17/19	89	0.4 polymyxin-B	1/16	6	0.5	15/20	75
0.005	7/15	47	2 lysozyme	15/15	100	1	2/10	20

Lysozyme abolished the pyrogenicity of ten times the amount of the minimum pyrogenic dose of the bacterial polysaccharide, while no such effect appeared after incubation with the other cationic macromolecules. However, the concentration of the bacterial polysaccharide had to be sufficiently high (20 $\mu\text{g./ml.}$) and the incubation had to proceed for a sufficient length of time (1–2 weeks) to obtain consistent and large decrease in pyrogenicity. Bacterial polysaccharide (20 $\mu\text{g.}$) and commercial lysozyme (16 mg.) were incubated together in 1 ml. of water at 37° for different lengths of time; Fig. 1 gives the average fever curves obtained with various dilutions of this mixture. The control curves were obtained with solutions of the bacterial polysaccharide incubated at 37° without lysozyme.

The presence of sodium polyglucose sulphate abolished lysozyme's ability to reduce the fever-producing activity of the bacterial polysaccharide. When lysozyme (16 mg.) and sodium polyglucose sulphate (24 mg.) were admixed in this ratio (which was known to inhibit completely the enzyme activity; Mora & Young, 1959), and this mixture was then incubated with the bacterial polysaccharide (20 $\mu\text{g.}$) in 1 ml. water, the fever-producing activity of the bacterial polysaccharide was retained even after incubation for 1 or 2 weeks at 37° (Fig. 2). Apparently the polyglucose sulphate complexed with the lysozyme, as indicated by the appearance of white turbidity, and the action of the lysozyme on the bacterial polysaccharide was inhibited.

Lysozyme decreased the tumour-damaging effect of the bacterial polysaccharide, but to a smaller extent: to about one-half of the original bacterial polysaccharide activity after 2 weeks' incubation at 37°. The bacterial polysaccharide itself did not lose activity after similar incubation.

Thus, the effect of lysozyme on the bacterial polysaccharide was different from the other cationic macromolecules, and indicated that two processes might be

involved: first, an immediate effect, probably due to macromolecular interaction, and secondly, a slower, probably enzymic reaction. The following experiments were carried out to clarify the nature of the lysozyme—bacterial polysaccharide interaction.

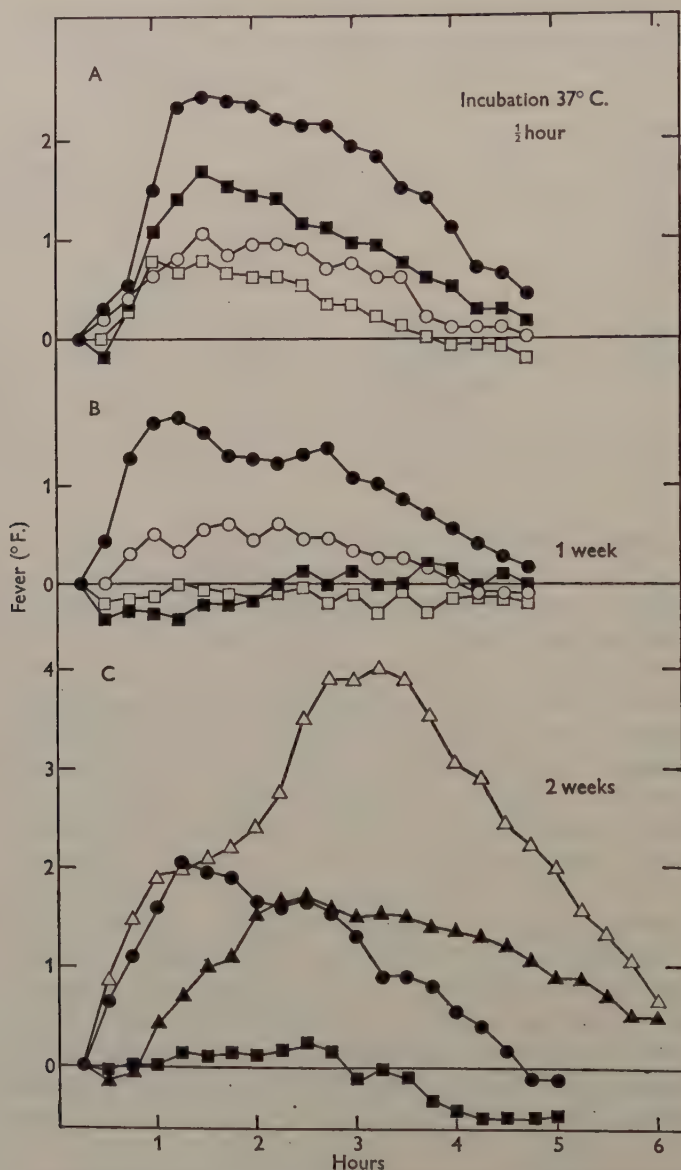


Fig. 1. Pyrogenicity in rabbits of the bacterial polysaccharide after different periods of incubation (37°) with lysozyme. Each point represents average fever from four rabbits after injection. A. Incubation $\frac{1}{2}$ hr., \circ = 0.01 μ g. polysaccharide/rabbit, \square = 0.01 μ g. polysaccharide + 8 μ g. lysozyme, \bullet = 0.1 μ g. polysaccharide, \blacksquare = 0.1 μ g. polysaccharide + 80 μ g. lysozyme. B. The same after incubation for 1 week. C. After 2 weeks incubation, \triangle = 1 μ g. polysaccharide, \blacktriangle = 1 μ g. polysaccharide + 800 μ g. lysozyme; \bullet and \blacksquare as in A.

The bacterial polysaccharide (10 mg.) was titrated at 0° in aqueous solution with 'basic lysozyme'. Figure 3 shows the changes in pH and in optical density. Neutralization occurred after 1.5–2 times as much lysozyme as polysaccharide was added, and also in this same range the optical density (turbidity) increased at a rapid rate. After 28 mg. lysozyme had been added (see arrow in Fig. 3) an aliquot of the suspension was set aside for further studies (see below). The titration was

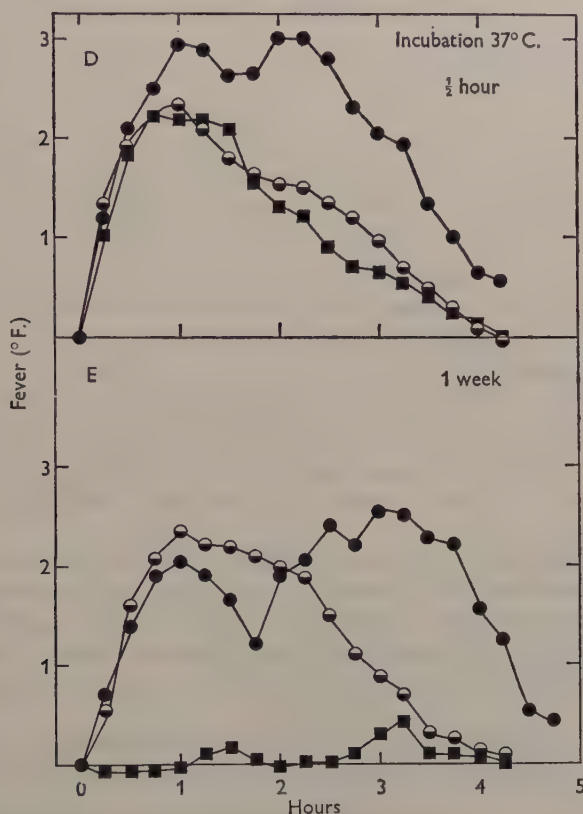


Fig. 2. Inhibition by polyglucose sulphate of lysozyme's ability to decrease pyrogenicity. D. Incubation $\frac{1}{2}$ hr., 37°, ● = 0.1 μ g. bacterial polysaccharide, ■ = 0.1 μ g. polysaccharide + 80 μ g. lysozyme, ⊖ = 90 μ g. lysozyme + 120 μ g. polyglucose sulphate sodium salt + 0.1 μ g. polysaccharide. E. The same after 1 week incubation. Notice that the effect of lysozyme (■) was inhibited by polyglucose sulphate (⊖). Results were similar after 2 weeks incubation.

continued on the remainder but there was no indication of further neutralization or precipitate formation (dashed line, Fig. 3). When sodium chloride was added to the final turbid suspension containing the excess lysozyme (establishing 0.1 N-NaCl conc.) the precipitate rapidly dissolved ($D = 0.154$) indicating dissociation of the complex. Similar immediate clearing occurred after adding a few drops of N-NaOH ($D = 0.076$, pH = 12.7).

One part of the aqueous suspension titrated to the point indicated by the arrow in Fig. 3 was centrifuged to collect the insoluble precipitate. To another part of the

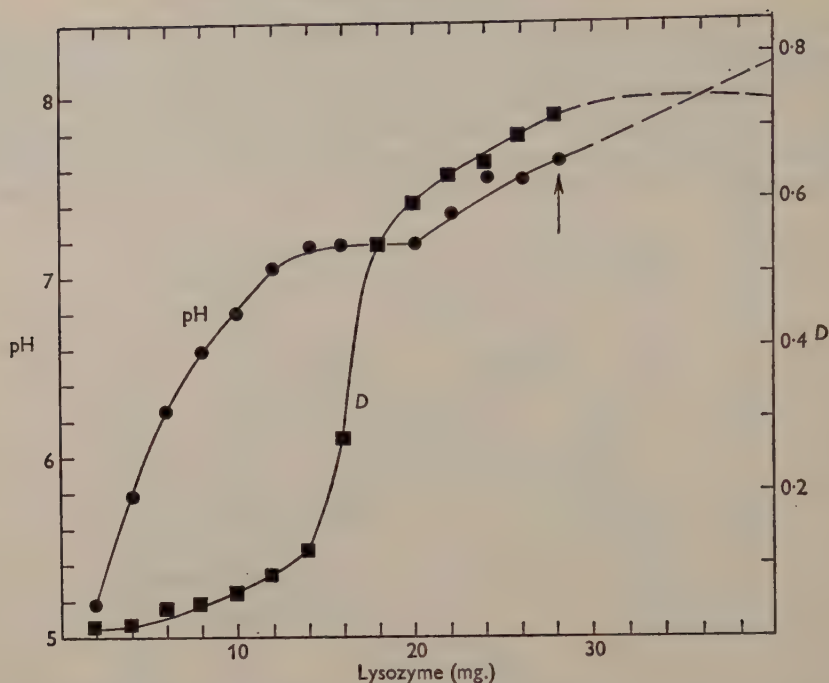


Fig. 3. Titration of bacterial polysaccharide (10 mg.) with 'basic lysozyme' in aqueous solution at 0° ; changes in pH, and in optical density (D) at $640\text{ m}\mu$.

suspension solid sodium chloride was added to establish 0.154M -concentration before centrifugation, thereby bringing about a dissociation by the salt similar to that which would be expected upon injection into body fluids. The supernatant fluids and the sediments from both the aqueous and from the saline solution were then analysed chemically for bacterial polysaccharide and for lysozyme concentration, and also

Table 2. *Interaction of bacterial polysaccharide with basic lysozyme in aqueous and in saline solutions; separation of material and of biologic activity into soluble and sedimenting fractions*

	10 mg. bacterial polysaccharide titrated with 28 mg. basic lysozyme in aqueous solution							
	H ₂ O				Saline			
			pH 7.6					
	32,000 g		30 min.		32,000 g		30 min.	
	Supernatant		Sediment		Supernatant		Sediment	
	Chemical	Bio-assay*	Chemical	Bio-assay	Chemical	Bio-assay	Chemical	Bio-assay
% recovery								
Bacterial polysacch.	81	50	58	50	78	100	22	0
Lysozyme	51	24	57	24	84	60	16	0

* Bioassay of bacterial polysaccharide by tumour-necrotic potency. Determinations were carried out in serial dilutions and on sufficient tumour-bearing animals to make the estimate valid $\pm 10\%$.

Bioassay of lysozyme by enzymic potency, see p. 83.

tested biologically for tumour-damaging potency and for lysozyme activity on *M. lysodeicticus* (Table 2).

The sediment obtained from the preparation in water contained about half of the bacterial polysaccharide with unimpaired tumour-damaging potency. Half of the lysozyme was also in this sediment, but the enzymic activity decreased to about one-quarter of the total. In the supernatant fluid the bacterial polysaccharide had high potency, while lysozyme activity was only one-half of what would have been expected on the basis of the chemical concentration, indicating that a soluble complex was present, but without decrease of the tumour-damaging activity of the bacterial polysaccharide in such a complex.

In saline solution, 22 % of the bacterial polysaccharide was present in the sediment without having demonstrable tumour-damaging activity, but still all the starting tumour necrotic potency was accounted for in the remaining 78 % concentration in the supernatant fluid. Lysozyme enzymic activity was somewhat decreased in the supernatant fluid, while there was no activity in the precipitate.

Dialysis experiments were carried out on aqueous and saline solutions of the bacterial polysaccharide interaction product with excess lysozyme, under conditions in which all the free lysozyme was demonstrated to be eliminated in a control experiment. Table 3 gives the final ratios in the supernatant fluids after dialysis against water and saline solution. Similar data are included on the dialysis of the interaction products of the bacterial polysaccharide and ribonuclease. In both aqueous and saline media the ratio of interacted (non-dialysable) bacterial polysaccharide to lysozyme or to ribonuclease was about one to two.

Table 3. *Dialysis of interaction products of the bacterial polysaccharide with basic lysozyme and with RNase in water and in saline solution*

	Interaction in water Ratio in dialysate* of bacterial polysaccharide to:		Interaction in 0.9 % NaCl Ratio in dialysate* of bacterial polysaccharide to:	
	Lysozyme	Ribonuclease	Lysozyme	Ribonuclease
Dialysed: against water	$\frac{1}{1.9}$	$\frac{1}{1.8}$	$\frac{1}{1.4}$	$\frac{1}{1.9}$
against saline	$\frac{1}{1.9}$	$\frac{1}{2.6}$	$\frac{1}{1.9}$	$\frac{1}{2.2}$

* The portion remaining after dialysis.

Partition cell analysis

Ultracentrifuge sedimentation of the bacterial polysaccharide shows a major component with 3.4*S* and several faster sedimenting components (6.9, 10.5 and 11.5*S*) of much higher molecular weights, with the second summit being at 10.5*S* (Fig. 4A).

Three partition cell analyses were carried out. In the first experiment the bacterial polysaccharide was centrifuged until all the higher molecular weight components passed into the lower half of the cell, but some of the slowest sedimenting material was retained in the upper half (Fig. 4B). In the second experiment the solution was centrifuged for a shorter time, which resulted in retaining also some of the faster sedimenting components in the upper half of the cell (Fig. 4C).

In the third experiment an interaction product of bacterial polysaccharide and lysozyme was centrifuged.

When the bacterial polysaccharide (10 mg.) interacted in aqueous solution (1 ml.) with basic lysozyme (28 mg.), as in the titration experiment above, a precipitate formed, which largely dissolved when the sodium chloride concentration was brought to 0.154M. The small amount of residual insoluble material was separated by low speed centrifugation (2150 g) and the supernatant was kept for 16 hr. at 4°. After this the supernatant fluid had only two slow-moving components in the analytical cell, with 1.92S and 3.86S, and no faster sedimenting components (Fig. 4D).

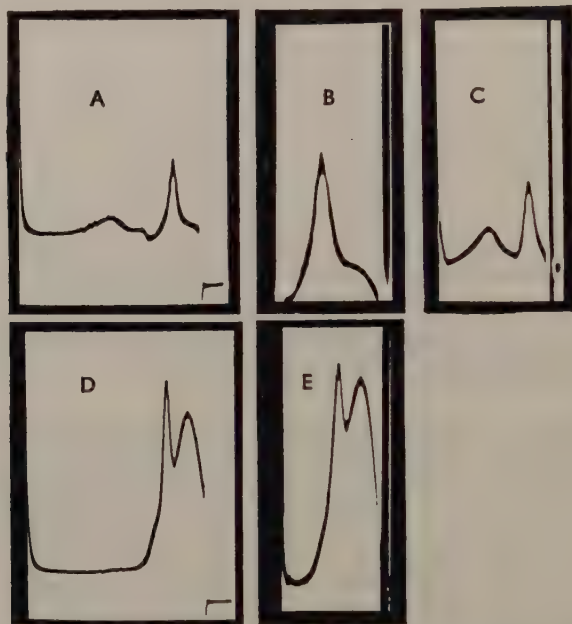


Fig. 4. Sedimentation of bacterial polysaccharide and of the complex with lysozyme in the ultracentrifuge. In all experiments bacterial polysaccharide conc. was 1% in 0.154M-NaCl; temp. 25°; centrifugation at top speed (59,780 rev. min.). Sedimentation from right to left. A. Bacterial polysaccharide in analytical cell after 30 min. B. Same in the moving partition cell. Photograph taken during deceleration at 2000 rev. min. after the moving partition re-occupied its median position; the lower half of the cell is dark because the light is blocked out by the supports of the partition. Total effective sedimentation time corrected to top speeds 66 min. C. The same. Total effective sedimentations time 20.70 min. D. Bacterial polysaccharide and lysozyme (orig. conc. 2.8%) after 30 min. at top speed. E. The same in the moving partition cell during deceleration at 2000 rev. min. Total effective sedimentation time 41.27 min.

In the third partition cell experiment the supernatant from the low-speed centrifugation of the bacterial polysaccharide + basic lysozyme mixture was sedimented to the extent shown in Fig. 4E.

In each partition experiment solutions from the upper and lower half of the cells, careful saline rinses of the lower halves of the cells and also uncentrifuged controls were refrigerated, and the next day were assayed for tumour-damaging and for fever-producing activity. Results are summarized in Table 4.

In the first partition experiment (Fig. 4B) at a dilution which in the uncentrifuged control would have given seventy times the ED₅₀, the supernatant had no detectable tumour-damaging activity; and also had no pyrogenic activity at a dilution which would have had 1200 times the MPD. All of the tumour-damaging activity and a large amount of the fever-producing activity were recovered, however, from the lower part of the cell. The appearance of the schlieren pattern indicated that a considerable amount of the slower sedimenting component (3.4S) was still present in the supernatant. Chemical determination was not possible because of the low level of the bacterial polysaccharide.

Table 4. *Fractions of the total biological activities recovered from compartments of the partition cell*

These values were determined by serial dilution and finding the approximate ED₅₀ values for the tumour-damaging activity and the MPD values for pyrogenicity and by comparing these to the controls.

Expt. (Fig.)	1 (4 B)		2 (4 C)		3* (4 E)	
	Tumour	Fever	Tumour	Fever	Tumour	Fever
Supernatant fluid	$< \frac{1\dagger}{70}$	$< \frac{1\dagger}{1200}$	$\frac{1}{7}$	$\frac{1}{7}$	$\frac{1}{20}$	$\frac{1}{20}$
Subnatant fluid	$\frac{6}{7}$	Pyrogenic†	$\frac{5}{7}$	$\frac{5}{7}$	$\frac{10}{20}$	$\frac{10}{20}$
Rinse	$\frac{1}{7}$	Pyrogenic†	$\frac{1}{7}$	†	$\frac{4}{20}$	†
Plug removed before centrifuging	—	—	—	—	$\frac{0.5}{20}$	Approx. $\frac{1}{2}\dagger$

* Bioassays carried out 1–6 days after the ultracentrifugation.

† Sufficient experiments were not carried out to find the ED₅₀ or the MPD value, since the available concentration was insufficient.

These results indicated that the active components sedimented rapidly and that the slow sedimenting component (3.4S) was not pyrogenic, and that it was also not tumour damaging, when the assays were carried out at the maximum available concentration.

In the second partition experiment where some of the more rapidly sedimenting material remained in the supernatant fluid (Fig. 4C), the supernatant fluid had about one-seventh of the original tumour damaging and also of the pyrogenic activity, while the residual activities were found in the lower half of the cell. On the basis of the bioassays of the supernatant fluid, sedimentation constant calculations by the method of Yphantis & Waugh (1956*a, b*) gave a figure of $15.7 \pm 0.5S$ for both the tumour-damaging and fever-producing activity.

In the third partition experiment (Fig. 4E), in which the bacterial polysaccharide-lysozyme solution was sedimented, bioassays showed that the fraction of the original tumour-damaging and pyrogenic material which remained in the top half of the cell was $1/20$, giving a calculated sedimentation constant of $8.7 \pm 0.3S$ for both of

these activities. In the insoluble material, which was removed before the partition cell analysis, there was only one-fortieth of the tumour-damaging potency, while the pyrogenic activity was high.

DISCUSSION

The tumour-damaging activity of the anionic bacterial polysaccharide was decreased when it was mixed in aqueous solution with typical cationic macromolecules, such as ribonuclease or polymyxin B. Furthermore, this reduction was annulled when an anionic macromolecule (polyglucose sulphate), with stronger dissociating groups and higher negative charge density than the bacterial polysaccharide, was added. These phenomena indicated direct complexing, and uncoupling, of charged macromolecules. In the first step the cationic macromolecules presumably blocked the sites, or components, in the bacterial polysaccharide responsible for its tumour-necrotizing potency. In the second, the stronger anionic polyglucose sulphate formed complexes with the cationic macromolecule, and thus liberated the tumour-damaging site of the bacterial polysaccharide. This mechanism is similar to the one we proposed on the reversible inhibition of enzymes (Mora & Young, 1959), and on the blocking of toxicity (Mora, Young & Shear, 1959).

Lysozyme, however, did not decrease the tumour-damaging activity of the bacterial polysaccharide at first; on the contrary, the tumour-damaging activity was somewhat enhanced when lysozyme and the bacterial polysaccharide were incubated for short periods. Apparently the complexing in this instance was with different components, or sites, of the bacterial polysaccharide, from those involved in the case of the other cationic macromolecules. There was no doubt of immediate complex formation: titration and precipitation curves (Fig. 3) indicated neutralization at a weight ratio of 1.5–2 lysozyme to bacterial polysaccharide, and the lysozyme was present in about the same ratio in non-dialysable complex both in water and in saline (Table 3). Electrostatic complexing with lysozyme must have left the tumour-damaging component or site free to exert its biological action. Addition of polyglucose sulphate to the complex of lysozyme and the bacterial polysaccharide decreased the tumour-damaging effect below that of the bacterial polysaccharide control. The fever-producing component was concentrated in the insoluble interaction product, recovered by low-speed centrifugation (Table 4, expt. 3).

Lysozyme was different from other cationic macromolecules in another respect, it reduced pyrogenicity of the bacterial polysaccharide but only through a slow process, taking 1–2 weeks to show a marked effect. Pyrogenic activity of the bacterial polysaccharide at ten times the minimum pyrogenic dose was completely abolished, and a considerable reduction of fever activity occurred even at 160 times the minimum pyrogenic dose (cf. Fig. 1C). It was necessary to have a relatively high bacterial polysaccharide concentration during incubation. These two features (a slow process, and high substrate concentration increasing the rate of the process) suggest that the reduction of pyrogenic activity occurred through an enzymic process, probably through slow breakdown of certain portions of the bacterial polysaccharide. This enzymic type of activity of lysozyme was blocked by polyglucose sulphate, as expected on the basis of our previous work on lysozyme inhibition (Mora & Young, 1959).

The above findings suggested that different sites or species of the bacterial polysaccharides were responsible for the tumour-damaging and for the fever-producing activity.

Two approaches were used to investigate further the heterogenicity of the bacterial polysaccharide. One was partial precipitation with lysozyme and the study of distribution of the tumour-damaging activity between the supernatant and the sediment upon centrifugation; the other was partition cell analysis of both tumour-damaging and pyrogenic activities.

The precipitation and centrifugation method indicated that about 22% of the bacterial polysaccharide, which sedimented in saline solution, was devoid of, or at least, had very low, tumour-damaging activity; it also confirmed the fact that both soluble and insoluble complexes are formed with lysozyme in aqueous solution (cf. Table 2).

The results of the partition cell analysis of the bacterial polysaccharide showed that both the pyrogenic and the tumour-damaging activities were associated with components which had a high sedimentation rate ($> 10S$), and which probably were large macromolecular complexes. The major component of the bacterial polysaccharide ($3.4S$) was not pyrogenic in the maximum dose tested ($1200 \times \text{MPD}$). This component also had lower tumour-damaging activity than the original ($< 1/70$). The limitations of the available quantities for tumour-damaging assay and the limited sensitivity of this assay does not allow further conclusion from this experiment.

The faster sedimenting components did not show up in ultra-centrifugation after the treatment with lysozyme (Fig. 4D). Apparently, these were the components which were preferentially precipitated, and also probably these were the ones digested eventually by the lysozyme. The supernatant fluid of the interaction product showed two slow sedimenting components (Fig. 4, D and E). One of these might be a breakdown product, the other an association complex of the slow sedimenting component with lysozyme.

The ultracentrifuge experiments did not give information on the actual size of the active components. It is possible that the sites which were responsible for pyrogenic or tumour-damaging activity were of much smaller size than the sedimentation constant indicates, and they were only incorporated rather firmly in a larger macromolecular entity which, when broken down by hydrolysis, resulted in complete loss of the biological activities.

It should be kept in mind that the enzymic type of digestion of the high molecular weight fever-producing components was not completely selective, since after 2 weeks' incubation there was also some decrease of tumour necrotic activity, to about one-half of that of the original polysaccharide. Also, our data do not justify the assumption of completely independent physico-chemical entities, one type being purely pyrogenic, the other tumour-damaging. The separation and the different behaviour of the functions was demonstrated, of course, only to the limits of the sensitivity of our biological assays.

The above reported experiments, however, indicated: that two of the biological activities (the pyrogenic and the tumour-damaging activity) of the bacterial polysaccharide complex from *Serratia marcescens* probably are caused by different physico-chemical entities; and that they behave differently in macromolecular

interaction and separation of them is a possibility in an active form when macromolecular size is retained. However fractionation on a preparative scale should be carried out to prove this last point.

The original observations on pyrogenicity were carried out at the suggestion of, and in collaboration with, Dr F. Rosen. Dr W. Carroll and Dr H. Kahler helped with the ultracentrifuge experiments. Mr Ellis Sheets provided experimental assistance. We would like to thank Dr M. J. Shear for his interest throughout this work.

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Spore Formation and 'Dimorphism' in the Mycobacteria

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SUMMARY

In studying 8 strains of *Mycobacterium tuberculosis* and 7 strains of atypical mycobacteria all 15 were found to produce, in addition to the typical acid-fast cells, non acid-fast ones, which gradually developed intracellular spore-like bodies; later free-lying spores were seen in the same cultures. This process occurred in heavily inoculated Löwenstein-Jensen medium cultures, which were at least 8 weeks old and were frequently aerated during incubation. With the atypical mycobacteria it occurred more readily in cultures in Kirschner fluid medium than on solid media. When the cultures containing spores were inoculated on nutrient agar plates, endospore-forming, rapidly growing organisms were obtained, which were not acid-fast. These organisms when obtained from independent cultures of the same strain appeared to be identical in bacillary and colonial morphology at their first isolation on nutrient agar, but the organisms from different strains showed variation in these characters. Thus mycobacteria appear able to grow in two different forms: (a) form 1, which is acid-fast and multiplies by fission only; (b) form 2, which is not acid-fast, produces endospores regularly and can be maintained in pure culture on nutrient agar. A series of phases of development of form 2 cells in the cultures of form 1 organisms in serial smear examination of Löwenstein-Jensen medium cultures is described. It is suggested that mycobacteria might be considered as dimorphic organisms in the same sense as some of the human pathogenic fungi are known to be dimorphic. Evidence is submitted that form 2 organisms are not contaminants.

INTRODUCTION

During experiments on the bacillary morphology of Mycobacteria (Csillag, 1960, 1961) and *Nocardia asteroides*, smears containing structures which resembled bacterial endospores or some sorts of fungal spores were occasionally seen. Such structures were found in the cultures of 17 of 24 strains of *Mycobacterium tuberculosis*, 20 of 42 strains of atypical mycobacteria (8 of group I, Runyon, 1959; 1 of group II; 7 of group III; 4 of group IV) and all of 4 strains of *N. asteroides*. When cultures containing these 'spores' were inoculated on nutrient agar plates, pure growths of spore-bearing bacilli or coccobacilli were obtained within 2 days, none of which were acid-fast. At first these organisms were considered as contaminants, but the possibility that they were derived from the acid-fast mycobacteria and nocardia was suggested by the following observations: (1) There were differences in the morphology of the spore-bearing, non acid-fast forms obtained from the different species of mycobacteria and nocardia. Thus, strains of *M. tuberculosis* always yielded Gram-

negative organisms, whereas the atypical mycobacteria and nocardia strains yielded bacilli which were Gram-variable or Gram-positive. (2) The spore-bearing organisms obtained from different cultures of the same strains appeared to be identical. (3) The spore-bearing forms were isolated repeatedly in spite of precautions taken to avoid contamination. Systematic experiments were therefore carried out to establish whether these organisms were derived from, and represented a phase in, the life cycle of the acid-fast mycobacteria. The results are reported here. No systematic investigations have been done on the strains of *N. asteroides*. For purposes of convenience, the acid-fast mycobacteria are termed form 1 and the spore-bearing organisms, which are not acid-fast, form 2.

METHODS

Mycobacterium tuberculosis

Organisms used. Seven strains of I 968, I 971, I 977, I 1053, I 1122, I 1133, I 1155 were recently isolated from sputa of seven newly diagnosed and untreated British patients with pulmonary tuberculosis. The isolates were sensitive to isoniazid, streptomycin and *p*-aminosalicylic acid, and were fully virulent in the guinea pig shortly before the *in vitro* experiments were started. The isolation of these strains and the sensitivity tests were made by methods described elsewhere (Tuberculosis Chemotherapy Centre, Madras, 1959). One laboratory strain, H37 Rv. Atypical mycobacteria: Group I (Runyon, 1959; photochromogens): 4 strains (260, 266, 353, 1438). Group III (Runyon, 1959; Battey-type): 3 strains (223, 248, 585). All these seven strains were obtained from Dr E. H. Runyon (Veterans Administration Hospital, Salt Lake City, Utah, U.S.A.). They were all obtained from sputa, gastric contents or resected lung material of patients with pulmonary disease. All strains of *M. tuberculosis* and atypical mycobacteria were maintained on Löwenstein-Jensen medium at 37°, and were subcultivated at intervals of a few months.

Media. (1) The Löwenstein-Jensen medium used was without potato starch (Jensen, 1955). (2) 7H-10 oleic acid-albumin agar plates (Cohn, Middlebrook & Russell, 1959). (3) Kirschner medium (Mackie & McCartney's *Handbook*, 1960) and Kirschner-base glycerol medium (containing 10% (v/v) in place of 2% (v/v) glycerol) dispensed in 3 ml. amounts in $\frac{1}{2}$ -oz. screw-capped bottles. (4) Nutrient agar prepared by addition of 1.4% (w/v) agar to meat-extract+peptone broth (Oxoid No. 2, Oxo Ltd., London). All solid media were incubated at 37° for 4 days and all fluid media for 5-6 days before use, as a test of their sterility.

Cultivation. All cultures were incubated at 37°. Plates of 7H-10 medium and nutrient agar were sealed in polythene bags during incubation. All bacteriological manipulations were carried out in an inoculation cabinet.

Aeration of cultures. Provision of an additional air supply to the form 1 cultures growing on Löwenstein-Jensen medium was carried out as follows. The medium was normally dispensed in 3 ml. amounts in $\frac{1}{2}$ -oz. screw-capped bottles, and incubated with the caps firmly on. Aeration was carried out by flaming the outside of the cap, loosening and flaming it again, removing the cap for a few seconds, flaming the neck of the bottle and replacing the cap. Slopes of Löwenstein-Jensen medium were also prepared in 'aeration tubes' which consisted of a test tube fitted with a rubber

bung through which was passed a 3 cm. diam. glass tube plugged with cotton wool. The tube was attached to 3 mm. length of rubber tubing closed with a clip. The clip was opened for 30 min. to allow the entry of air. During each batch of aeration a nutrient agar plate and a 5% horse blood agar plate were exposed near the hands of the worker in the inoculation cabinet in an attempt to cultivate air-borne organisms.

Staining methods. Smears were allowed to dry under an ultraviolet lamp for 8–10 min., and fixed for 1 min. in methanol. Jensen's method of Gram staining (Mackie & McCartney, 1960) was modified by decolorizing rapidly with acetone and counterstaining with dilute (1/15) carbol fuchsin. Control smears of *Staphylococcus pyogenes* and *Escherichia coli* were usually stained on the same slide. In the Ziehl-Neelsen staining (Mackie & McCartney's *Handbook*, 1960) smears were decolorized with acid ethanol (NaCl 20 g.; distilled water, 500 ml.; ethanol, 99.3% (v/v), 1500 ml.; HCl conc., 20 ml.) for not less than 5 min. and counterstained with Loeffler's methylene blue for 5–10 min. The Loeffler's methylene blue was at least 5 weeks old. Staining for spores was done with warm 1% (w/v) malachite green in 1% (w/v) phenol in water for 5 min., rinsing in tap water for 3 min., and counterstaining in 1% (w/v) aqueous safranin for 30 sec.

RESULTS

Production of form 2 mycobacteria

Origin of form 2 from form 1 organisms. The following experiment was carried out to obtain form 2 organisms from cultures of form 1 mycobacteria. A total of 15 strains (8, *Mycobacterium tuberculosis*; 4, group I atypical; 3, group III atypical) were plated out on 7H-10 medium plates and, after incubation for 17 days, single colonies were plated out again. With each strain, 6 slopes of Löwenstein-Jensen medium were each heavily inoculated from a separate well-isolated colony grown on the second plate for 17 days. An equal number (90) of Löwenstein-Jensen medium slopes from the same batch of medium were 'inoculated' in a similar manner, but with a sterile loop. These control slopes were subsequently subjected to exactly the same procedures as their counterpart slopes which had been inoculated with living organisms. The slopes were inoculated in a random order.

Groups of slopes containing one slope inoculated with a colony from each strain and an equal number of control slopes inoculated with a sterile loop were subjected to different procedures which are set out diagrammatically in Fig. 1. The slopes of groups A1 and B1 were in aeration tubes, while all the remaining slopes were in screw-capped bottles. The procedures for groups A and A1 were otherwise identical, and similarly for groups B and B1. All cultures were incubated for 14–24 weeks. In groups A, A1, B, B1 and C the cultures were aerated for portions of the incubation period, with the aim of investigating the relationship between aeration and the occurrence of form 2 organisms. Cultures of group D were not aerated. At 14 weeks cultures of group B1 were subcultured on to fresh Löwenstein-Jensen medium slopes (group S) and, after incubation for 8 weeks, the growth from group S was again subcultured into tubes of Kirschner and Kirschner-base glycerol medium (groups K and Kg). Further control slopes or tubes inoculated with a sterile loop were also included in groups S, K and Kg.

At the times indicated in Fig. 1, samples of the initial inoculum and of the growth in the cultures were inoculated in two nutrient agar plates and examined in smears stained by the Ziehl-Neelsen methods. The nutrient agar plates were incubated for 5 days and colonies were examined and smears were made from them daily. After the end of the experiment each Löwenstein-Jensen medium culture in group C was re-incubated for a further 3 months and was aerated twice weekly. At the end of this period smears were stained from the growths on these slopes.

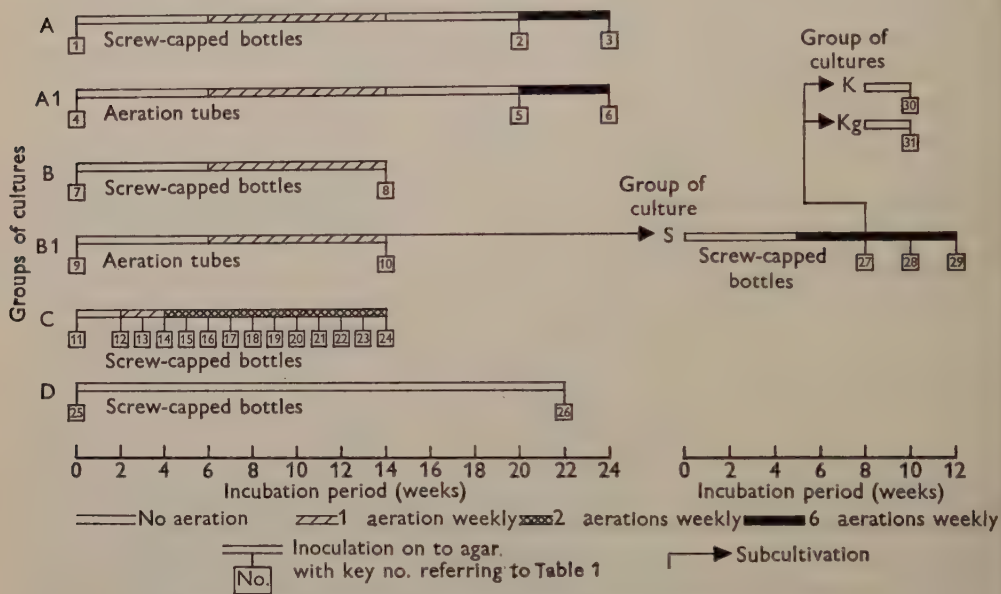


Fig. 1. Incubation and aeration of form 1 mycobacteria cultures.

The results of the cultures on nutrient agar plates inoculated at the intervals indicated in Fig. 1, from the growths on Löwenstein-Jensen and Kirschner media, are set out in Table 1. All samples of the colonies on 7H-10 medium plates used for inoculating the Löwenstein-Jensen medium slopes failed to yield growth when inoculated on to nutrient agar plates. All 15 strains yielded form 2 colonies on nutrient agar plates on 3 to 10 occasions of sampling. Form 2 colonies were obtained only from form 1 cultures on Löwenstein-Jensen medium which had been incubated for 8 weeks or more. An isolation from a culture was not always followed by further isolations from the same culture, possibly because the samples were usually taken from a small area on the slope and were not representative of the entire growth. At each isolation a heavy growth of at least 100 form 2 colonies was obtained on incubation of the nutrient agar plates for 1-2 days. None of the control slopes, which had been initially inoculated with a sterile loop yielded any growth on the nutrient agar plates. None of the cultures of group D, which were not aerated, yielded form 2 colonies, when they were sampled at 22 weeks. However, form 2 colonies were obtained on each occasion of sampling at 8-14 weeks from 3 to 8 cultures in group C, which were being aerated twice weekly.

Of the 88 pairs of nutrient and blood agar plates exposed for cultures of airborne

Table 1. Isolation of form 2 organisms from form 1 mycobacteria

Group of cultures	Period of incubation (weeks)	Key no. from Fig. 2	<i>M. tuberculosis</i>								Atypical mycobacteria						
			H37 Rv	<i>M. tuberculosis</i>							Group I				Group III		
				I 968	I 971	I 977	I 1053	I 1122	I 1133	I 1155	260	266	353	1438	223	248	585
A	0	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	20	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	24	3	+	—	—	—	—	—	+	+	—	—	—	—	—	—	—
A1	0	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	20	5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	24	6	—	+	+	—	—	—	+	+	—	—	—	—	—	—	—
B	0	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	14	8	—	+	+	+	—	+	+	—	—	—	—	—	—	—	+
B1	0	9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	14	10	+	+	+	+	—	+	+	—	—	—	—	—	—	—	+
C	0	11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	2	12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	3	13	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	4	14	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	5	15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	6	16	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	7	17	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	8	18	+	—	—	—	—	—	—	—	—	—	—	+	—	+	—
	9	19	+	—	—	—	—	+	+	—	+	—	+	+	+	+	—
	10	20	—	—	—	—	—	+	—	—	+	—	+	+	+	—	—
	11	21	—	+	—	—	+	—	—	+	—	—	—	+	+	+	—
	12	22	—	—	—	+	—	+	—	—	+	—	—	+	—	—	—
	13	23	+	—	—	—	—	+	—	—	—	+	+	+	—	—	—
	14	24	—	—	+	—	+	—	—	—	+	—	+	+	—	—	+
D	0	25	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	22	26	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S	8	27	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	10	28	—	—	—	—	+	—	—	—	—	—	—	+	+	—	—
	12	29	+	+	—	—	—	—	—	—	—	—	+	+	+	—	—
K	2	30	—	—	—	—	—	—	—	—	—	+	—	—	—	—	+
KG	2	31	—	—	—	—	—	—	—	—	+	+	+	+	+	—	+

— indicates no growth of samples on nutrient agar plates.

+ indicates growth of form 2 colonies on nutrient agar plates.

contaminants during the aeration of the cultures, only two plates yielded growth on subsequent incubation, and the colonies on both consisted of Gram-positive cocci.

After incubation for 2 days, plates bearing form 2 colonies of the first isolate from each strain were formalized for comparison with the other isolates. The bacillary morphologies of the form 2 organisms were compared in smears, stained by the Ziehl-Neelsen methods, when endospores first become apparent, that is, after the nutrient agar plates had been incubated for 1–5 days. Both the bacillary and colonial morphologies of the form 2 organisms, isolated from the same form 1 strain on different occasions from the same culture and from different cultures appeared identical. The similarity of the bacillary morphologies of five pairs of form 2

organisms, each member of the pair being obtained from a different group, and the variation from strain to strain are illustrated in Pl. 1, figs. 2-6.

The effect of aeration. Aeration of the Löwenstein-Jensen medium cultures appeared to be necessary for the development of the form 2 organisms in the form 1 cultures, as seen with the cultures of group D, which were not aerated and did not yield form 2 colonies. It also appears probable that the interruption of aeration resulted in the form 2 organisms losing their ability to grow on nutrient agar. In groups B and B1 at 14 weeks, following an 8-week period of aeration form 2 colonies were obtained from 13 of the 30 cultures. However, cultures in groups A and A1 were aerated in the same way until the 14th week; they were not aerated for 6 weeks, when sampling failed to yield any form 2 colonies, but after a further period of intensive aeration 7 of the 30 cultures yielded these colonies. Subculturing followed by a period of growth without aeration also appeared to prevent the isolation of form 2 colonies. Colonies were obtained from 7 of the 15 cultures in group B1 at 14 weeks, when they were subcultivated as group S. After growth without aeration for 5 weeks and with aeration for a further 3 weeks, none of the cultures of group S yielded form 2 colonies.

The effect of different media. The effect of different media on the development of form 2 mycobacteria appeared to differ according to whether the form 1 strains were *Mycobacterium tuberculosis* or atypical mycobacteria. Form 2 colonies were obtained from sampling of 7 of the 8 strains of *M. tuberculosis*, but from only 1 of the 7 strains of atypical mycobacteria in the Löwenstein-Jensen cultures of groups A, A1, B and B1. Again, the subcultures in Kirschner-base glycerol medium (group Kg) yielded form 2 colonies from none of the 8 strains of *M. tuberculosis*, but from 6 of the 7 strains of the atypical mycobacteria.

Morphology of form 2 organisms in form 1 cultures

Phases of development. The phases of development of form 2 organisms are described as they appeared in the cultures of group C (Table 1), since in this group smears were made at weekly intervals. Samples of the initial inoculum and of the growth in the cultures were stained by the Ziehl-Neelsen method. The microscopic appearances are illustrated diagrammatically in Fig. 2, since the early phases were difficult to see and even more difficult to photograph. Some of the phases appeared together in the smears and it is therefore difficult to be certain that the order of development is exactly as described. Considerable variation occurred from culture to culture in the time intervals between the occurrence of the phases, and only the average time is reported.

Form 2 cells first appeared between the fourth and sixth week of incubation as just visible non acid-fast dots lying free among the form 1 bacilli (Fig. 2, phase 1). At about 6 weeks short rods with one pointed end and a polar granule at the other, appeared, giving the impression of a germination tube (Fig. 2, phase 2). At about 8 weeks, slender rods with pointed ends were seen, and these were either stained evenly or contained a single deeper stained granule located centrally, or two granules located at the poles (Fig. 2, phase 3). A minority of these cells were arranged parallel to each other. Between 9 and 14 weeks the intracellular granules often became larger than the rest of the cell, distorting the cell wall and giving the impression of an early stage of intracellular spore-formation. At 14 weeks a pro-

portion of the cultures yielded smears which contained either one or both of the following forms: (a) evenly and deeply stained rods with squared ends, often lying in chains or parallel (Fig. 2, phase 4a); (b) free-lying oval spores, either isolated or in clumps (Fig. 2, phase 4b). An example of phase 4b is shown in Pl. 1, fig. 1. All of the cultures in group C (Table 1) at the end of the additional 3-month period of incubation and aeration yielded smears containing organisms in phases 4a and b.

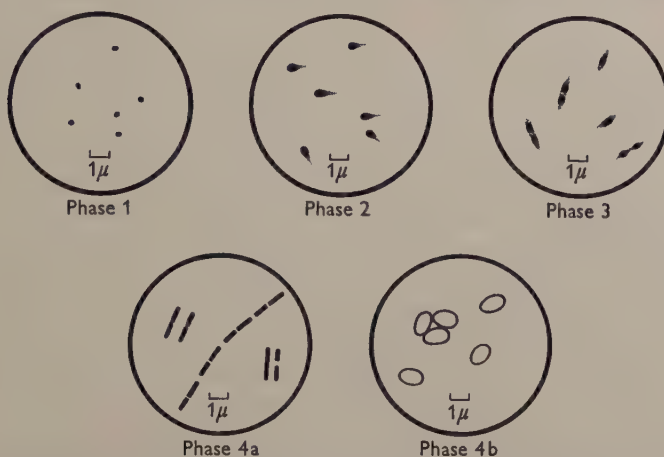


Fig. 2. Diagrammatic illustrations of development of form 2 organisms in form 1 cultures.

Form 2 organisms were stained in their early phases with difficulty, and did not stain with an aqueous solution of methylene blue or with Loeffler's methylene blue when the latter was less than 5 weeks old. The vegetative forms were never acid-fast. The central area of the free spores was usually unstained, but occasionally weakly acid-fast; the walls were stained blue. The spores stained green with safranin-malachite green. The number of the form 2 organisms in proportion to the number of form 1 organisms was highly variable. In phases 1 and 2, form 2 organisms were present only sparsely and could easily be overlooked. When in phase 3, the ratio of their number to the number of form 1 organisms was not less than 1:100.

Changes in the culture medium. The Löwenstein-Jensen medium did not alter until the form 2 organisms were in phases 1 and 2; when they were about to change from phase 3 to phases 4a and 4b the medium became yellowish, particularly at the edges of the slopes. When form 2 organisms reached phase 4, the growth often developed a brownish pigmentation or liquefied the medium, giving the impression that it was contaminated.

Ability to grow on nutrient agar. Form 2 organisms in phases 1, 2 and early in phase 3 did not grow on nutrient agar. In phase 3, when the granules became large enough to distort the cell wall, and in phase 4a and 4b, colonies were invariably obtained on nutrient agar plates. Form 2 organisms in phases 1, 2 and early 3 disappeared from smears and lost their ability to grow on nutrient agar when aeration of the Löwenstein-Jensen medium cultures was interrupted for a long period, as described above. Once form 2 organisms reached phase 4, they were not

influenced by interrupting the aeration, as shown by a further experiment in which cultures contained form 2 organisms in phase 4b and were incubated without aeration for 12 months. The spores remained unchanged and yielded colonies on nutrient agar.

Growth on Löwenstein-Jensen medium. When form 1 cultures containing form 2 organisms in phases 1-3 were subcultured to fresh Löwenstein-Jensen medium, normal growth of form 1 organisms appeared and the growth did not contain form 2 organisms. However, when form 1 cultures on Löwenstein-Jensen medium, containing form 2 organisms in phases 4, were subcultured on the same medium, the medium of the subculture turned yellow or brown and was often softened. Form 1 organisms never grew on it and at most, scanty colonies of form 2 were obtained on it.

Properties of form 2 organisms isolated on nutrient agar

Bacillary morphology. The bacillary morphology of the form 2 organisms is described from smears prepared from the colonies of the first isolate on nutrient agar. The appearance in smears stained by the Ziehl-Neelsen method are described when endospores first became apparent (1-5 days). Gram staining is described in smears taken at 2 days, irrespective of sporulation. Staining with safranin-malachite green was done when the cultures contained numerous free spores.

The bacillary morphology of individual strains is not described in detail, because further experience suggests that it is profoundly affected by the nature of the medium, the period of incubation, the age and previous history of the inoculum, and the temperature of incubation. Nevertheless, it is important to emphasize that the bacillary morphology of the different isolates from the same form 1 strain always appeared identical at the first isolation on nutrient agar. The form 2 organisms obtained from 12 strains were rods (Pl. 1, figs. 2, 3, 5, 6), which varied from strain to strain in their size (range $0.5 \times 3.0 \mu$ - $1.0 \times 2.5 \mu$), in the shape of their ends, in the arrangement of cells, in the location of their endospores and in the extent to which the spores distorted the cell walls. None of the rods was acid-fast. The rods from *M. tuberculosis* were Gram-negative, those from the atypical mycobacteria were Gram-variable or Gram-positive. After longer periods of incubation (3 days-2 weeks) small, Gram-positive granules appeared within some of the rods from all strains. The free spores were oval (size range $0.7 \times 1.0 \mu$ - $1.2 \times 1.5 \mu$), their cell walls were blue in the Ziehl-Neelsen smears and Gram-positive, the central area was usually unstained, but occasionally weakly acid-fast and Gram-negative. They were uniformly green when stained with safranin-malachite green. The form 2 organisms obtained from the remaining three strains (H37 Rv, I 977, I 1133) were cocci of very uneven size and shape, often arranged in chains (Pl. 1, fig. 4), which were not acid-fast and were Gram-negative. The large, oval forms seen in Pl. 1, fig. 4, were not as typical of spores as those seen in the rod-shaped form 2 organisms, but they were still considered as spores since they stained green with safranin-malachite green. These spores stained uniformly Gram-negative. Although these coccoid forms were obtained from all of the 3 strains at all isolations, it has been observed that strain H37 Rv yielded rod-shaped form 2 organisms, with typical endospores after prolonged incubation and aeration on Löwenstein-Jensen medium. This finding suggests that the coccoid form may be an intermediate stage followed by a final rod-shaped stage similar to the remaining strains of *M. tuberculosis*.

Viability. The form 2 organisms which were composed of cocci all died out after 1-2 subcultivations on nutrient agar. With the remaining strains, subcultures of colonies which did not contain free spores had a similar poor viability. When free spores were present, subcultures on nutrient agar could often be maintained in a viable state at room temperature for 2 months, but during regular subcultures at intervals of 2 months for a year most have died out.

Colonial morphology. The morphology of the colonies of the form 2 organisms is described from the first isolate from each strain obtained on nutrient agar plates after incubation for 2 days. The colonies were examined at $\times 10$ magnification with a plate microscope. As with bacillary morphology, no attempt has been made to describe the detailed colonial morphology of each strain since the appearances were markedly variable on further subcultures. Each strain, however, yielded the same colonial type when first isolated from the Löwenstein-Jensen medium slopes. The colonies obtained from the *Mycobacterium tuberculosis* strains (Pl. 2, figs. 1, 2) were usually small (0.1-0.9 mm. diam.), but larger colonies (2.0-3.0 mm. diam.) were obtained from three strains. The colonies were usually discrete, but one strain produced a confluent thin film on the surface of the medium; they were circular, or irregular, effuse or raised, smooth or finely granular; some strains had colonies with one central umbo, or two umbos or were umbilicate; the edges were usually entire though the large colonies had crenated or slightly fimbriate edges; all colonies were grey and opaque, most were friable, but the larger colonies were butyrous; one strain had colonies firmly adherent to the medium. The colonies obtained from the atypical mycobacteria were larger (3.0-8.0 mm. diam.); they were circular or irregular, smooth, glistening, butyrous either with a uniform structure and entire edges (Pl. 2, figs. 3, 4) or a beaten-copper, worm-cast surface with rhizoid edges (Pl. 2, fig. 5).

DISCUSSION

Evidence that the form 2 organisms were derived from the form 1 organisms and were not contaminants can be derived from the following observations. (1) There was variation in the bacillary and colonial morphologies of form 2 organisms from strain to strain and from species to species, yet these characters were closely similar in multiple isolations from the same form 1 strain. It will be appreciated that the form 1 cultures in groups A, A1, B, B1 (in this context B1 includes its subcultures S, K and Kg) and C, were each derived from a separate colony on 7H-10 medium plates (Fig. 1). The similarity of the bacillary morphologies of five pairs of form 2 organisms, each member of the pair being obtained from a different group and the variation from strain to strain are illustrated in Pl. 1, figs. 2-6. Some of the strains of *Mycobacterium tuberculosis* and atypical mycobacteria were again examined for the production of form 2 organisms in further experiments. The form 2 isolates had colonial and bacillary morphologies identical to those described here. (2) The conditions under which form 2 organisms were obtained, such as length of incubation and type of medium, were different with strains of *M. tuberculosis* and of the atypical mycobacteria. If the form 2 organisms arose as airborne or medium contaminants then a random pattern of these characters would be expected and would not be related to the species of the form 1 strains; however, the observations reported provide strong evidence against this view, particularly since the inoculation and aeration of the cultures was done in a random order. (3) The controls incorporated

in the experiments also provided evidence that the form 2 organisms did not originate from the air or the culture medium. The failure to obtain form 2 organisms from the media 'inoculated' with a sterile loop and aerated during the course of the experiment is against these organisms arising from the air or the medium. The failure to obtain any colonies resembling the form 2 organisms on the plates exposed in the inoculation cabinet during aerations suggest that they did not result from aerial contamination. The failure to obtain form 2 colonies from those slopes which were not aerated (series D, Fig. 1) suggest that they were not medium contaminants. (4) Evidence that contamination arising during aeration of the Löwenstein-Jensen medium cultures was not responsible for the isolation of form 2 organisms is provided furthermore by comparing the frequency with which form 2 colonies were obtained from cultures aerated either by removal of their screw-caps or by allowing air to enter through a cotton-wool plug. Form 2 colonies were obtained from 9 of 30 cultures in groups A and B, which were aerated by removal of their caps and in 11 of 30 cultures in groups A1 and B1, which were set up in aeration tubes (Fig. 1, Table 1). (5) Although most of the form 2 organisms resemble the genus *Bacillus* when grown on nutrient agar, all of the isolates from *M. tuberculosis* strains were definitely Gram-negative and it would be remarkable to obtain such a high proportion of Gram-negative *Bacillus* species. Further experiments, to be described elsewhere, show that the form 2 strains have a complex morphology and life-cycle resembling but not identical with certain species of the families Actinomycetaceae and Streptomycetaceae (Waksman & Henrici, 1943).

The existence of form 2 organisms in the form 1 cultures used as the inoculum is improbable. The Löwenstein-Jensen medium slopes were inoculated with form 1 cultures which had been purified by single colony selection on two occasions. If a form 2 organism had survived this purification it should have been visible in the smears or should have grown in the cultures on nutrient agar which were made from the inocula in these experiments.

As considered above, it is reasonable to conclude that form 2 organisms were derived from the form 1 cells in certain stages of their life cycle. The exact stage at which the transition between these two forms occurs is uncertain. The serial examinations of Löwenstein-Jensen medium cultures (group C) suggest that in the early phases of their development the form 2 organisms are not able to grow on nutrient agar. In the late phases of their development when spores were present either in an advanced stage in the cells or were lying free, positive isolates were obtained from the spores. These structures were considered as spores since they resemble the spores of different micro-organisms (Bacillaceae, Streptomycetales) in their morphological and staining characteristics and in their capability to survive for longer periods than the vegetative cells. Heat resistance of spores was not investigated, since it is known that spores of Streptomycetales are reproductive bodies rather than resistant bodies and are destroyed by heat at 60–65° (Waksman, 1950). Spore formation (conidia) in organisms closely related to mycobacteria has been known for a long time. The order Actinomycetales (Waksman & Henrici, 1943) contains five genera (*Mycobacterium*, *Actinomyces*, *Nocardia*, *Streptomyces*, *Micromonospora*) of which two, *Streptomyces* and *Micromonospora* have been known for long to form a certain type of spores (conidia), while spores have recently been described in a third genus, *Nocardia*, by Gordon & Mihm (1958). The close relationship between the

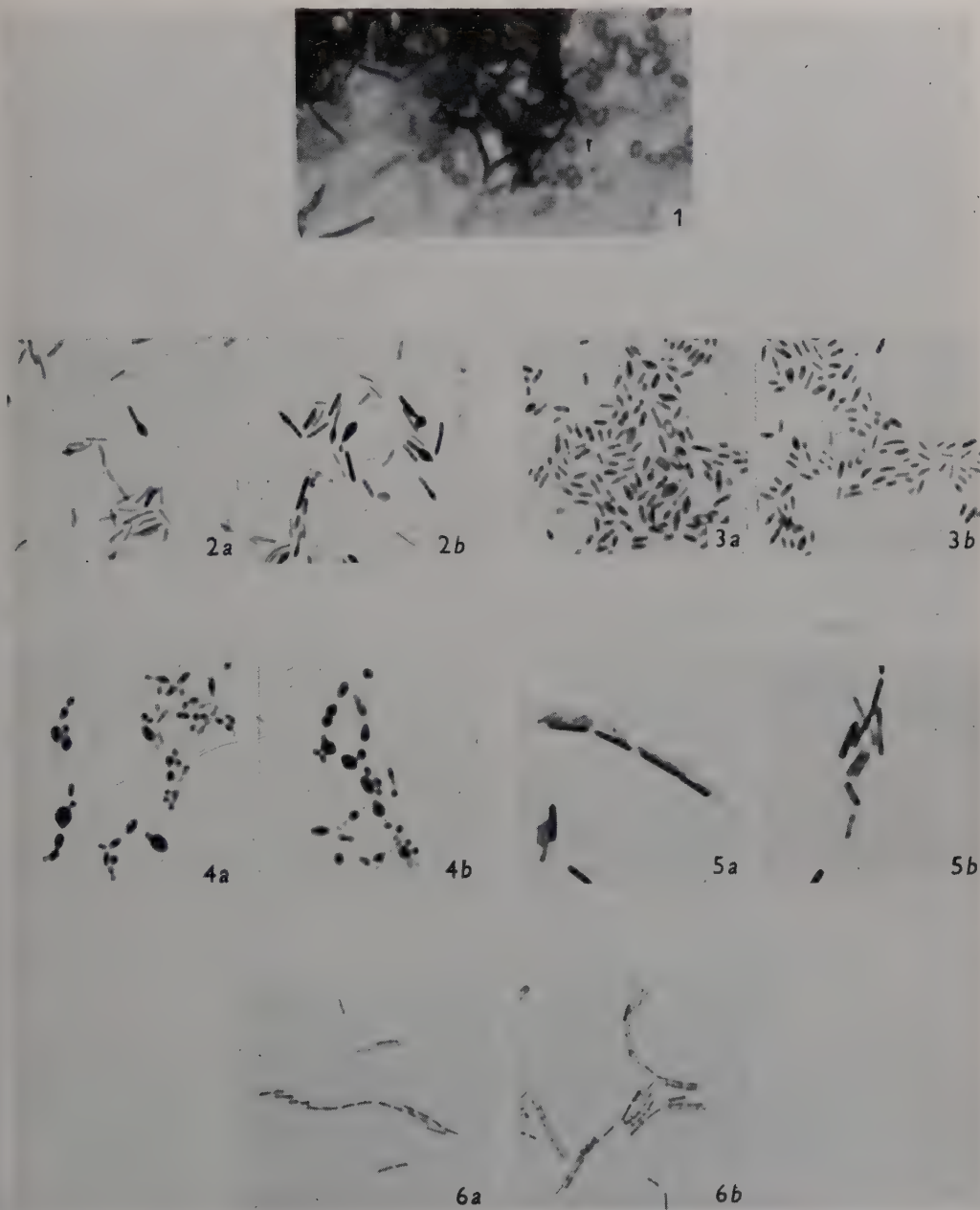
genera *Mycobacterium*, *Actinomyces* and *Nocardia* has been commented on by many authors (Jensen, 1931; Skinner, Emmons & Tsuchiya, 1947; Gordon & Mihm, 1957; Cummins & Harris, 1958). Spore formation of Mycobacteria was described and illustrated by Xalabarder (1954) and by Brieger & Glauert (1956). The latter authors observed round bodies in the filaments of an avian strain of *Mycobacterium tuberculosis* by electron microscopy; these bodies were identified as spores and had a structure similar to the spores of *Bacillus cereus* and *B. megatherium*. The existence in acid-fast mycobacteria cultures of forms that are not acid-fast, is extensive, and has been reviewed by Bassermann (1953) and Koelbel (1957). Rosenthal & Heagan (1955) described by using bright field, phase and electron microscopy certain cells in the culture of *M. tuberculosis* strain BCG which correspond in bacillary morphology and staining properties to the description of form 2 organisms reported here. They considered that the bacilli which correspond most closely to the early phase 3 described here reverted to normal acid-fast bacilli. In Lack & Tanner's (1953) paper illustration of organisms in mycobacteria cultures closely resembling the phases 3 and 4b of the present paper are shown in Pl. 3, fig. 10, and Pl. 1, fig. 3. The authors found that these forms were not acid-fast (Dr C. H. Lack, personal communication) except for the middle of the spore-like structures in Pl. 1, fig. 3, which they considered as abnormal nuclei.

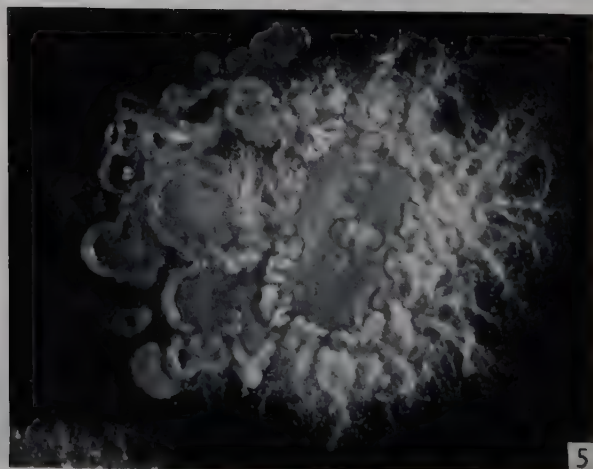
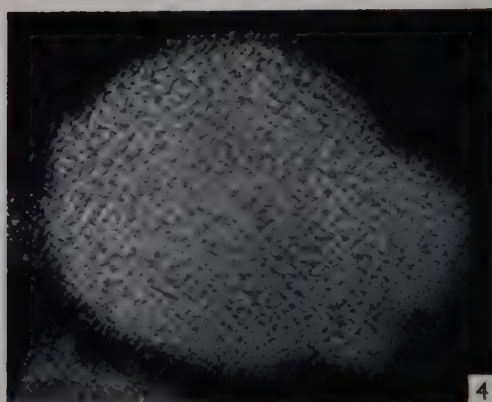
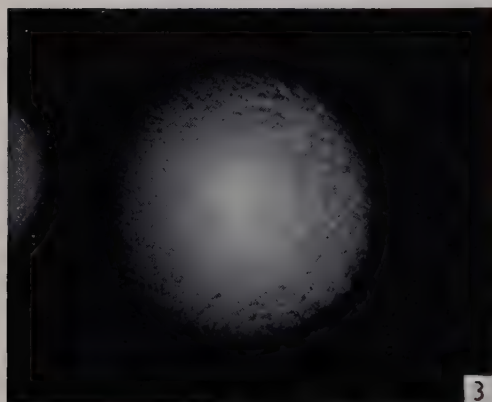
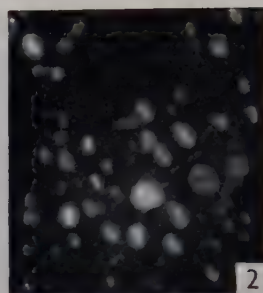
Although form 2 organisms differ from form 1 organisms in a variety of important characters such as bacillary morphology, staining characters, colonial morphology, speed of growth and nutritional requirement, they seem to be not two different organisms but two different forms of the same organism. In mycology some species are known to exist in two entirely different forms (dimorphism); this phenomenon is considered by Langeron & Vanbreuseghem (1952) as a special manifestation of polymorphism. In the pathogenic dimorphic fungi, the morphology of the parasitic form is usually simple but the saprophytic form is complex. For example, *Histoplasma capsulatum* always grows in the host as a budding yeast, but *in vitro* it grows either as a yeast, producing smooth, glistening colonies, or reverts to a hyphal type of growth with cottony, mould colonies in which chlamidospores occur regularly. It is reasonable to suggest that the forms 1 and 2 of the mycobacteria described here are examples of such dimorphism. There appears to be no previous report of proved dimorphism in the mycobacteria. Since the form 2 organisms when in phase 4 and when growing on nutrient agar are similar to strains of the genus *Bacillus*, it would seem probable that form 1 cultures containing them would usually have been discarded as contaminated. The results of attempts to convert form 2 to form 1 mycobacteria and a fuller description of the characteristics of form 2 organisms will be described at a later date.

I wish to express sincere thanks to Dr D. A. Mitchison for his interest in this work and for his severe criticism which nevertheless proved extremely valuable. Our stimulating discussions contributed much to the soundness of the experiments. Finally, I am grateful to him for reading this paper. I am also grateful to Mr K. Robinson and Miss Janet Lloyd for their conscientious technical assistance.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. *Mycobacterium tuberculosis*: 1-6 weeks' incubation with frequent aeration, on Löwenstein-Jensen medium. Form 1 cells and free spores are shown. Ziehl-Neelsen stain. $\times 2000$.

Figs. 2-6. Form 2 organisms isolated on nutrient agar plates, incubated for 1-5 days. Pairs of photographs of the same strain; the 2 members of the pairs having been obtained in separate groups (Fig. 1). Ziehl-Neelsen stain. $\times 1500$.

Fig. 2a. *M. tuberculosis*, strain I971, group B.

Fig. 2b. *M. tuberculosis*, strain I971, group B1.

Fig. 3a. *M. tuberculosis*, strain I1155, group A.

Fig. 3b. *M. tuberculosis*, strain I1155, group A1.

Fig. 4a. *M. tuberculosis*, strain I1133, group A.

Fig. 4b. *M. tuberculosis*, strain I1133, group A1.

Fig. 5a. Group I, atypical, strain 260, group C.

Fig. 5b. Group I, atypical, strain 260, group Kg.

Fig. 6a. Group I, atypical, strain 1438, group C.

Fig. 6b. Group I, atypical, strain 1438, group C.

PLATE 2

Figs. 1-5. Colonies of form 2 organisms at their first isolation on nutrient agar plates, after 2 days' incubation. $\times 10$.

Fig. 1. *M. tuberculosis*, strain I968.

Fig. 2. *M. tuberculosis*, strain I971.

Fig. 3. Group I, atypical, strain 260.

Fig. 4. Group I, atypical, strain 1438.

Fig. 5. Group III, atypical, strain 223.

Further Observations on Changes in the Phage-Typing Pattern of Phage Type 80/81 *Staphylococci*

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SUMMARY

Two distinct temperate phages (594a, 594n) lysogenizing phage type 80/81 *staphylococci* and producing gains in sensitivity to typing phages 52 and 52A were found in lysogenic clones of a strain of *staphylococcus* of phage type 52/52A/80/81. When either of these phages infected type 80/81 cocci a new phage (594b) appeared in the lysates. This phage was unable to replicate in type 80/81 cocci but could grow in them when they had been lysogenized with phage 594a or 594n. The source of phage 594b was found to be recombination of phage 594a or 594n with a prophage present in cocci of phage type 80/81. This prophage was completely defective and could be demonstrated only by recombination. The gains in phage sensitivity that follow lysogenization of type 80/81 strains with the so-called 'converting' phages can be most satisfactorily explained on the basis of prophage substitution.

INTRODUCTION

Asheshov & Rippon (1959) and Rountree (1959) described changes in the phage-typing pattern of *staphylococci* of phage type 80/81 occurring after lysogenization with certain temperate phages, which were called 'converting' phages for want of a better name. The change of particular interest was that by which the lysogenized cocci became sensitive to the typing phages 52 and 52A so that their typing pattern became 52/52A/80/81. The mechanism of this change remained obscure. By replica plating Rountree demonstrated the presence in old broth cultures of the propagating strain of phage 80 (P.S. 80) of mutants sensitive to phages 52 and 52A and she suggested that the converting phages might select such pre-existent mutants, which might be more readily lysogenized than the wild type. On the other hand, Asheshov & Rippon found no mutants sensitive to phages 52 and 52A in their type 80 strain. Furthermore, when they had converted this strain by lysogenization and then 'cured' it of the converting phage by ultraviolet irradiation, the cured strain remained sensitive to phages 52 and 52A. In addition both the original strain and the cured strain gave identical lytic and lysogenic responses to a converting phage. These observations indicated that neither lysogenic conversion nor selection of pre-existing mutants could explain the phenomenon. Prophage substitution

remained a possibility, but all attempts to demonstrate lysogenicity in cocci of phage type 80/81 were unsuccessful. It has now been found, however, that type 80/81 staphylococci contain a completely defective prophage and that the change in the typing pattern after lysogenization with one of the converting phages is due to substitution of this prophage by the converting phage.

METHODS

Phage type 80/81 staphylococci. Seven staphylococcal strains typing as 80/81 and isolated in a number of different localities were chosen for study. A strain was considered to be phage type 80/81 if it was lysed only by these two phages amongst the 21 phages comprising the basic set of typing phages, both when typing was carried out with the phages at the routine test dilution (RTD) and with the phages at 1000 times greater concentration. The seven type 80/81 strains comprised the following: *P.S. 80*, the propagating strain for phage 80, isolated in Sydney in 1953. A streptomycin-resistant mutant of *P.S. 80* was used in some experiments. It will be referred to as *P.S. 80^{sr}*; *P.S. 81*, the propagating strain for phage 81, isolated in Canada in 1956 (Bynoe, Elder & Comtois, 1956); *U9*, isolated in Philadelphia, Pa., U.S.A. and received from Dr J. Baldwin, Columbus, Ohio, in 1956; *8428*, isolated in Oamaru, New Zealand in 1954; *1406/1*, obtained from Miss K. Key of the Commonwealth Serum Laboratories, Melbourne, in 1957; *590*, isolated in Sydney in 1958; *6346*, isolated in England in 1958.

Phage type 52/52A/80/81 staphylococci. Four strains of this phage type were used. They were as follows: *strain 594*, isolated from the same patient as strain 590 and presumed to have been converted from strain 590 *in vivo*. Lysogenic clones of this strain were used as the source of two converting phages and are described in detail below; *strain 6346 DL*, derived from strain 6346 which was lysogenized with a converting phage, 7287¹, and subsequently cured (Asheshov & Rippon, 1959). This strain is believed to be completely non-lysogenic; *strain 80 la*, a mutant of *P.S. 80* isolated by Rountree by replica plating from an old broth culture of *P.S. 80* which differed from the parent culture in being sensitive to the two typing phages 52 and 52A (Rountree, 1959); *strain 1406/2*, presumed to have been derived from 1406/1 (type 80/81) during mouse passage and differing from the parent culture in being type 52/52A/80/81.

Preparation of phage stocks. Phage stocks were prepared either by ultraviolet (u.v.) induction of singly lysogenic strains or by propagation on a non-lysogenic strain in order to avoid contamination with phages carried by lysogenic strains. The method of u.v. induction was essentially that of Gorrill & Gray (1956). The cocci were grown with aeration in glucose veal broth for 2 hr., centrifuged and resuspended in phosphate buffer pH 7.0. They were irradiated with a Westinghouse 'Sterilamp' for 50 sec. at a distance of 38 cm., the suspension being agitated in a Petri dish during exposure. The irradiated cocci were inoculated into glucose veal broth at 5×10^7 cocci/ml. and incubated in a water bath at 37°. After 60 min. they were transferred to the bench where clearing of the culture took place within 2–5 hr. of irradiation. Such stocks had titres of the order of $1-5 \times 10^9$ infective particles/ml. In other cases phage stocks were prepared by propagating the phage on the non-lysogenic strain, 6346 DL, by the method of Swanstrom & Adams (1951).

Counting phages. Agar plates were flooded with a young broth culture of the

appropriate indicator strain of staphylococcus, the excess broth pipetted off and the plates allowed to dry before the phage dilutions in 0.01 or 0.02 ml. volumes were deposited on their surface. Three or four replicates were plated from each dilution and were not spread over the surface of the plate.

Lysogenization. This was carried out on agar as previously described (Rountree, 1959). All phages used for lysogenization were made by u.v. induction, filtered through Gradocol membranes of 0.8μ A.P.D. and tested for sterility before use. Some strains were lysogenized in broth, a quantity of phage theoretically sufficient to infect all cocci being added to young aerated cultures.

The lysogenized strains are described in the usual way, e.g. P.S. 80 (594a) or, more simply P.S. 80(a) is P.S. 80 lysogenized by the phage derived from strain 594A.

RESULTS

Phages isolated from strain 594

Staphylococcus strain 594, of phage type 52/52A/80/81, showed numerous plaques when grown on a phage-typing plate shortly after its isolation, and the original broth culture showed evidence of spontaneous clearing after a day at room temperature. It was plated on agar on two occasions and a number of entire single colonies picked after incubation. These clones, lettered A to R, were all of phage type 52/52A/80/81. All of the clones were lysogenic but three different phages were identified in the various clones. The behaviour of these three phages and of cocci lysogenized by them were valuable in demonstrating the mechanism of the conversion of type 80/81 strains to type 52/52A/80/81.

Phage 594a and 594n were converting phages present in the prophage state in clones 594A and 594N, respectively. Both belonged to serological group A and were inducible by u.v. radiation and by superinfection with the other phage; there was, however, no cross-immunity between them.

The third phage, 594b, was found in a single lysogenic clone, 594B. It also belonged to serological group A and was inducible by u.v. radiation and by superinfection with phage 594a or 594n. Cocci of P.S. 80 were immune to it but such cocci became sensitive to this phage after they had been lysogenized by either phage 594a or 594n and could then be used as an indicator for the phage. Clone 594G, doubly lysogenic for 594a and 594n could also be used as an indicator for phage 594b but was less sensitive than 594A or 594N. For the sake of brevity these three phages will be referred to subsequently as phage *a*, *n*, and *b*.

Table 1 summarizes the reactions of clones isolated from strain 594. Several representatives of each clonal type were identified. The *n* prophage in clone F was partly defective since no *n* phage was produced spontaneously and only a small amount when the clone was induced by u.v. radiation. The host range of the three phages was determined against a set of test strains of various phage types. Only minor differences were detected. The main difference between the three phages therefore lay in their failure to show cross-immunity and in the failure of phage *b* to lyse strains of phage type 80/81.

Origin of phage b

The origin of phage *b* and of clone 594B was examined. The working hypothesis that strain 594 was derived from a type 80/81 strain, 590, that had been infected

in vivo with the converting phages, *a* or *n*, did not explain the occurrence of phage *b* and clone 594B, since type 80/81 cocci (either P.S. 80 or 590) were immune to the phage. It seemed possible that phage *b* represented a mutant of phages *a* or *n*, sufficiently virulent to overcome the immunity of strains lysogenic for phages *a* or *n*, and which, at the same time, had mutated in such a way that it was no longer able to lyse cocci of P.S. 80. However, high-titre phage preparations made by u.v. induction of strains 594A and P.S. 80(*a*) and strains 594N and P.S. 80(*n*) showed no trace of phage *b* when tested on their homologous strains. That phage *b* arose as a mutant of phage *a* or *n* seemed unlikely. Another possibility was that phage *b* represented a hybrid phage resulting from recombination between phage *a* and phage *n*. Once again, however, high-titre preparations of phage *a* propagated on strain 594N and of phage *n* propagated on strain 594A failed to show the presence of phage *b*. When, however, either phage *a* or *n* was propagated on P.S. 80 or strain 590, phage *b* was regularly recovered in the lysates. It seemed, therefore, that phage *b* might have its origin in a hitherto undetected prophage in P.S. 80 or else be a recombinant of such a hypothetical prophage with the phage *a* or *n*.

Table 1. *Reactions of clones isolated from staphylococcus strain 594*

Clone no.	Sensitivity to phages			Lysogenicity for				Prophage state of clone		
	<i>a</i>	<i>b</i>	<i>n</i>	PS 80	PS 80(<i>a</i>)	PS 80(<i>n</i>)	594G			
594A	—	+	+	+	—	+	—	<i>a</i>	.	.
594N	+	+	—	+	+	—	—	.	.	<i>n</i>
594B	+	—	+	—	+	+	+	.	<i>b</i>	.
594C	—	—	+	+	+	+	+	<i>a</i>	<i>b</i>	.
594F	—	+	—	+	—*	+	—	<i>a</i>	.	<i>n_a</i> *
594G	—	+	—	+	+	+	—	<i>a</i>	.	<i>n</i>
594K	—	—	—	+	+	+	+	<i>a</i>	<i>b</i>	<i>n</i>

* = defective prophage, trace only on u.v. induction.

Lysogenicity of type 80/81 strains

Previous attempts to demonstrate lysogenicity in type 80/81 strains had been unsuccessful (Asheshov & Rippon, 1959; Rountree, 1959) but the appearance of phage *b* during the growth of phage *a* and *n* in these strains indicated that a further search should be made. When supernatant fluids of 2–4 hr. broth cultures of P.S. 80 or 590 were filtered and plated on 594A or 594N no plaques were formed. When, however, the supernatant fluids were tested before filtration, minute fuzzy plaques developed which were difficult to count even with a hand lens. When broth cultures were centrifuged for increasing lengths of time, the number of plaques appearing was found to be directly proportional to the number of unsedimented cocci of P.S. 80 in the inoculum. These results suggested that the appearance of plaques was intimately connected with the number of cocci of P.S. 80 able to develop on the surface of the plate. Furthermore, when the unfiltered supernatant fluid of a streptomycin-sensitive clone of P.S. 80 was plated on agar containing 1000 µg. streptomycin/ml. inoculated with the streptomycin-resistant strains P.S. 80^{sr}(*a*) or P.S. 80^{sr}(*n*), a situation in which the unsedimented P.S. 80 cocci were killed, no plaques were seen. When the supernatant of P.S. 80^{sr} was plated under these same conditions, phage plaques were visible, so that failure to see them in the platings from the strepto-

mycin-sensitive strains was not due to the phage itself being streptomycin-sensitive. Propagation of the phage which produced the fuzzy plaques yielded preparations identical in serological reactions and host range to phage *b*.

It was concluded that no free phage corresponding to phage *b* was produced by P.S. 80 cocci and that interaction between the converting phages and the type 80/81 cocci was necessary for its production. This interaction would occur on plates if a few type 80/81 cocci were deposited on a lawn of lysogenic cocci that spontaneously released some phage during incubation and thus infected the type 80/81 cocci. The most satisfactory explanation of these findings was that P.S. 80 cocci contained a completely defective prophage that could be detected only when it formed a recombinant with phage *a* or phage *n*. This prophage will be termed 80'. We have referred to phage *b* as a single entity regardless of whether it represents a recombinant between phage *a* and 80' or phage *n* and 80'. We have not yet detected any difference between these two recombinants and for the purpose of this paper no distinction will be made between them. A more detailed study of them is in progress.

Prophage substitution during conversion

If a defective prophage exists in type 80/81 cocci, it is clear that the changes in typing pattern that result from lysogenization might be explicable on the basis of prophage substitution. Type 80/81 cocci are immune to the recombinant phage, *b*, and also show 'blocking' of the typing phages 52 and 52A. After lysogenization with a converting phage, such as phage *a* or *n*, the cocci are sensitive to phages *b*, 52 and 52A. Thus the 80' prophage may determine the immunity to all three phages and its removal by lysogenization may lead to increased phage sensitivity of the cocci. Further evidence for the loss of defective prophage by substitution with a converting phage is derived from the fact mentioned above, that propagation of phage *a* in P.S. 80(*n*) and vice versa, failed to reveal the presence of any of the recombinant phage, *b*.

It follows that the spontaneous mutants of type 80/81 strains which are sensitive to phage 52 and 52A may have gained this phage sensitivity by spontaneous loss of the 80' prophage. Two such mutants were available, strains 80 1a and 1406/2. Both strains were sensitive to phage *b*. Neither strain yielded a recombinant phage when infected with phage *n*, and neither showed the minute fuzzy plaques when their supernatant fluids were plated on strain 594N. Similarly strain 6346 DL, which had been lysogenized with a converting phage and subsequently cured, was found to be fully sensitive to phage *b* and to be incapable of giving rise to it by recombination. It was concluded that all three strains had lost their defective prophage, the first two spontaneously, the latter by prophage substitution, and that this defective prophage determined the typing pattern of the type 80/81 strains and gave rise to the recombinant phage.

Origin of clone 594B

The origin of clone 594B, the singly lysogenic colony, containing only the *b* prophage, remained to be explained. It had been isolated from the original broth culture of strain 594 in which the recombinant phage was being produced. Since type 80/81 cocci are resistant to phage *b* they cannot be lysogenized by this phage. However, a spontaneous mutant which had lost its 80' prophage would be sensitive

and could be lysogenized. Alternatively, the doubly lysogenic colonies containing prophage *a* and *b* or *n* and *b* might be unstable and lose their *a* or *n* prophage.

The reconstruction of clones of various prophage states was attempted. Cocci of strain 590 were infected in broth with phage *a* or phage *n* and, after clearing, allowed to become turbid again. The culture was diluted and plated so as to obtain 100–200 colonies/plate after incubation. These master plates were replicated on to a plate flooded with strain P.S. 80 as an indicator of phage *a* or *n*, and on to either strain 594A or 594N, depending on which phage had been used for infection, as an indicator of phage *b*. By this means, colonies containing prophages *a* alone, *n* alone, *a* and *b*, and *n* and *b* were identified and isolated. Some of the colonies containing *a* and *b* or *n* and *b* were unstable when subcultured in broth and showed plaques when flooded on to agar plates, thus behaving like 'suicide' strains. Some single colonies picked from subcultures of these strains contained only the *b* prophage. Similarly, although doubly lysogenic colonies were readily obtained when clones 594A or 594N were treated with phage *b*, such cultures were sometimes unstable on continued subculture and cocci containing the *b* prophage alone could be isolated from them. The singly lysogenic clone 594B could therefore have arisen either by lysogenization of a spontaneous mutant or else by double lysogenization, first with a converting phage and second with the recombinant phage, and the subsequent loss of the 'converting' prophage.

Lysogenicity of other type 80 strains

All of the seven type 80/81 strains examined have yielded the recombinant phage after infection with phage *n*. Table 2 shows the count of phage *n* and phage *b* at the end of one-step growth experiments of phage *n* in five strains isolated from widely different places. All five strains were examined for the presence of free phage but none could be found and it was concluded that all contained the defective prophage. All changed their typing pattern to 52/52A/80/81 after lysogenization with phage *n*.

In addition, 85 strains which had been sent to the Staphylococcus Reference Laboratory in London for routine phage typing and which were phage type 80/81 when tested with the typing phages at the routine test dilution (RTD) were examined for carriage of the defective phage in the following way. The supernatant fluids of 24 hr. broth cultures of the strains were spotted on to plates flooded separately with strains 594A, 594N, 594B, and the non-lysogenic strain 6346 DL. The appearance of small fuzzy plaques on the first two strains and the absence of any reaction of the latter two strains was considered to be fairly good evidence of the carriage of the defective prophage 80'. Of the 85 strains 68 gave the expected result; 10 of the 68 were tested with the typing phages at 1000 RTD and all 10 were type 80/81. The supernatant fluids of the remaining 17 strains showed strong lytic reactions on all four indicator strains. On typing these strains at 1000 RTD all 17 were found to be type 52/52A/80/81, i.e. not typical 'phage type 80/81' strains. These results indicated that the carriage of the defective prophage 80' was characteristic of all typical type 80/81 strains.

Frequency of recombination

One-step growth experiments were run to determine the rate at which phage *a* or phage *n* recombined with phage 80' to form the recombinant phage *b*. A log-phase broth culture of P.S. 80 was spun down, washed once in phage adsorption medium

Table 2. *Production of phage 594b by staphylococcal strains of type 80 infected with phage 594n; counts made at the end of one step growth curves*

Strain no.	Phage 594n (particles/ml.)	Phage 594b (particles/ml.)
PS 80	1.7×10^9	5.9×10^8
PS 81	3.9×10^9	4.2×10^8
U9	2×10^9	7×10^8
8428	2.6×10^9	1.1×10^7
1406/1	6.4×10^9	2.7×10^8

(PAM; Hershey & Chase, 1952) and finally resuspended in PAM to a concentration of $1-2 \times 10^8$ viable units/ml. Sufficient phage *a* or *n* was added to infect about 40–50 % of the cocci in 15 min. at 37°. At the end of this time the adsorption mixture was centrifuged, washed once in PAM and finally resuspended in nutrient broth warmed to 37°, the time of resuspension being taken as 0 hr. Tenfold dilutions were made in warm nutrient broth to a point where 0.1 ml. contained about 1×10^2 infected cocci/ml. The dilution tubes were held at 37° throughout the experiment and at intervals samples were removed from the appropriate dilutions and titrated for phage *a* (or *n*) and for the recombinant phage. Titrations for the recombinant phage were made on agar containing 1000 µg. streptomycin/ml. The streptomycin-resistant indicator strain 80^{sr}(*a*) was used to titrate the recombinant phage where phage *a* was the infecting phage and strain 80^{sr}(*n*) where phage *n* was the infecting phage. In the presence of streptomycin, uninfected cocci of P.S. 80 which are deposited on the plate are unable to grow and no recombination can take place on the plate. Infected cocci in which recombination has taken place at the time of plating will probably produce a plaque, provided that at least one recombinant particle is already mature or matures within 2–3 min. of plating; cyanide-lysis experiments had shown that streptomycin at the concentration used required this length of time to exert its inhibitory effect. As a control on the streptomycin effect, titrations for phage *a* (or *n*) were made on nutrient agar and on streptomycin agar plates both flooded with strain 80^{sr}.

The results of a typical experiment are shown in Fig. 1. In this experiment phage *a* which was used to infect P.S. 80 had a latent period of about 35 min. and an average burst size of 20 particles of phage *a*/infected coccus. The actual burst size may be smaller since, staphylococci having a tendency to clump, the actual number of infected cocci during the latent period may be higher than the number recorded. Plaques of phage *a* on streptomycin agar began to appear at 5 min. and increased logarithmically up to 35 min., when the count equalled that on nutrient agar. This was interpreted to mean that mature particles of phage *a* appeared at 5 min. when some 3.4 % of infected cocci contained at least one mature phage particle. There was a delay in the appearance of mature recombinant particles until 20 min. At this time about 50 % of infected cocci contained mature *a* particles while only 0.0002 % contained a recombinant particle. The proportion of infected cocci containing recombinant particles increased rapidly until at 35 min. the proportion was about 0.07 %.

It was not clear from the results of a number of experiments whether or not the cocci that released the recombinant phage released more than one recombinant

particle. In most experiments the count of phage *b* continued to increase during the rise period to a final concentration approximately 2-3 times the count at the beginning of the rise period. This might represent a true burst size for the recombinant or it might be the result of late-lysing cocci contributing to the count of recombinant particles. When the infected cocci were lysed with cyanide during the latent period, curves such as that for phage *n* shown in Fig. 2 were obtained. Replication of phage *n* was complete at 50 min. but the release of the phage was not complete until 60 min. During this last 10 min. period the amount of phage *b* increased from $7.1 \times 10^5/\text{ml.}$ to $9.5 \times 10^5/\text{ml.}$ These results do not exclude the possibility of the replication of the recombinant in individual cocci. They do, however, suggest that the initial step of recombination occurs later in the latent period than the initial step of replication of the infecting phage which is in keeping with current knowledge of the recombination process in coli phages (Hershey, 1958).

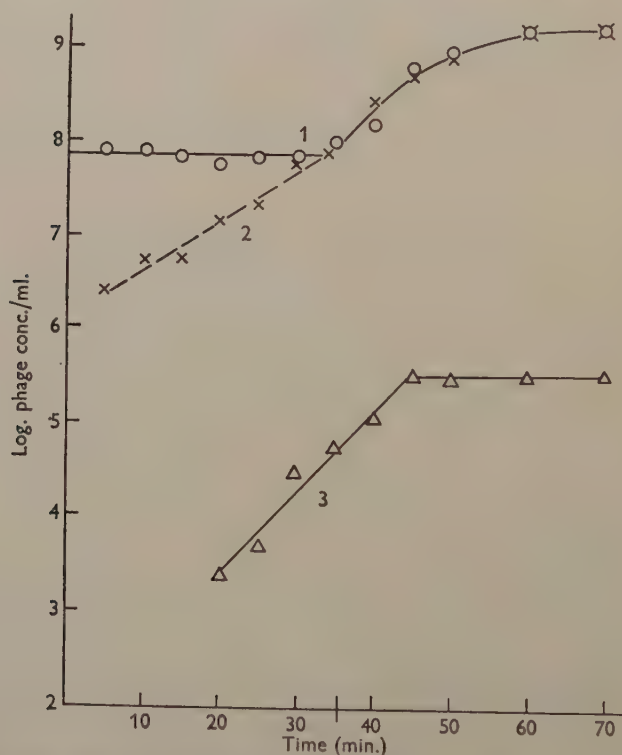


Fig. 1. One step growth curve of phage *a* in staphylococcus PS 80. Titrations for phage *a* were made on strain 80^{sr} on plain agar (curve 1) and on streptomycin agar (1000 $\mu\text{g./ml.}$) (curve 2). Titrations for the recombinant phage *b* were made on strain 80^{sr}(*a*) on streptomycin agar (curve 3).

The rate of recombination was calculated as the ratio of the number of infected cocci releasing the recombinant to the total number of infected cocci. However, without knowing whether the increase in the number of recombinant particles during the rise period represented an actual burst size or simply the contribution of late-lysing cocci it is impossible to know how many infected cocci actually released

at least one recombinant particle. If it be assumed that the recombinant replicates in those cocci that produce it and that the increase represents a true burst, the ratio would be best calculated on the number of cocci releasing any recombinant phage at the end of the latent period. If, on the other hand, the increase in the recombinant during the rise period be due entirely to the contribution of late lysing cocci, and no replication of the recombinant takes place in any coccus that releases it, then the ratio should be calculated on the total amount of the recombinant released at the end of the rise period.

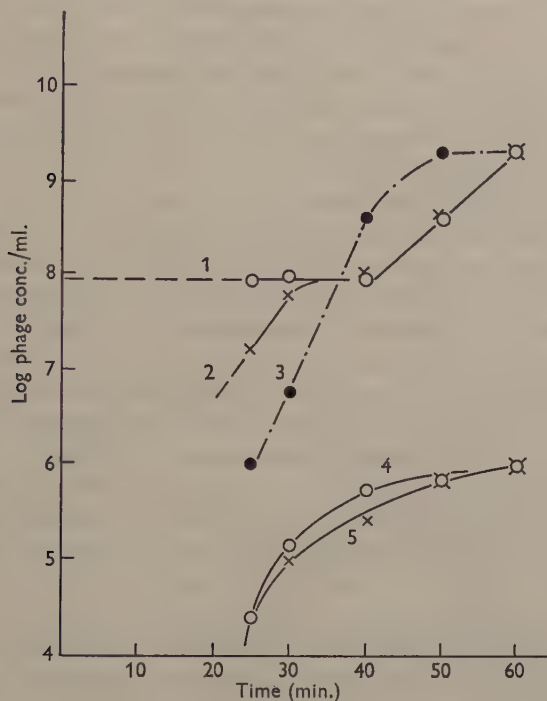


Fig. 2. Cyanide lysis experiment of *staphylococcus* PS 80 infected with phage *n*. Infected cocci were diluted into broth containing 0.02 M sodium cyanide after 25, 30, 40 and 50 min. These dilutions were held at 4° for 30 min. then at 37° for 60 min. before titrating for phage *n* on strain 80^{sr} and for the recombinant phage *b* on 80^{sr}(*n*). Curve 1 = phage *n* titrated on plain agar. Curve 2 = phage *n* titrated on agar containing 10 µg. streptomycin/ml. Curve 3 = phage *n* released by cyanide-lysed cocci at the time indicated. Curve 4 = phage *b* titrated on streptomycin (10 µg./ml.) agar. Curve 5 = phage *b* from cocci prematurely lysed by cyanide at the times indicated.

The figures obtained differed in different experiments but there was little difference regardless of whether phage *a* or phage *n* was used as the infecting phage. When calculations were based on the count of the recombinant at the end of the latent period (assuming a small burst size) the figures varied between 1/100 and 1/1000 with an average of about 1/600. When calculations were based on the final count of the recombinant (assuming no replication of the recombinant) the figures varied between 1/30 and 1/250 with an average of about 1/150.

DISCUSSION

The experimental results described in this paper are compatible with the hypothesis that the 'conversion' of type 80/81 staphylococci to type 52/52A/80/81 after lysogenization is due to prophage substitution. A similar example of prophage substitution in staphylococci was described by Gorrill (1957) who showed that a prophage carried by the propagating strain of typing phage 76 was displaced when the strain was lysogenized with phage 76. There are several similarities between the system studied by Gorrill and that investigated in the present paper. These include the instability of some of the doubly-lysogenic colonies and the gain in phage sensitivity following lysogenization. The main difference between the present system and that of Gorrill is the complete defectiveness of the 80' prophage. The prophage state in the type 80/81 cocci resembles that described by Cohen (1959) for the prophage of *Escherichia coli* B, which is completely defective and recognizable only by its ability to enter into recombination with phage P2.

The present results throw some light on to the relationship of the prophages in type 80/81 cocci. The presence of the 80' prophage causes a 'blocking' of the sensitivity of the cocci to infection with phages 52 and 52A. It may be postulated that this blocking is due to a steric interference by the 80' prophage which makes loci for phage 52 and 52A inaccessible to these phages. When the 80' prophage is displaced by the converting phages or by spontaneous loss, this steric interference disappears. The recombinant phage *b* can lysogenize converted type 80/81 cocci from which the 80' prophage has disappeared and this does not cause blocking of phage 52 and 52A. In other words, interference with phages 52 and 52A is a characteristic of 80' prophage but not of its recombinant. If, as Jacob & Wollman's work (1959) implies, attachment of the prophage to the bacterial chromosome may involve overlapping by the prophage of some genetic loci, then it may be postulated that the prophages *a*, *n* and *b* overlap fewer sites than does the 80' prophage.

The phages *a*, *n* and *b* are closely related. Work in progress shows that infection with any of them will induce the others when they are present as prophages. It was considered that phage *a* might be a 'dismune' (Bertani, 1958) mutant of phage *n* or vice versa. However, no evidence for such a mutation has been found. It must therefore be concluded that the original type 80/81 strain in the patient from whom the 594 clones were isolated was infected with two converting phages in the respiratory tract.

Cocci of type 80/81 infected with a converting phage in broth culture behave as suicide cultures. They survive the attack of the phage by becoming lysogenized with it; this, however, renders them sensitive to the recombinant phage which has been produced in a proportion of the infected cocci and on plating they will show plaques due to this phage. If such broth cultures are continued they may show a second clearing. The cocci surviving this second attack will be doubly lysogenic. Strains Bundaberg, 308, 313 and 315 which had been previously used as sources of converting phages (Rountree, 1959) were examined and found to be resistant to phages *a* and *b* but sensitive to phage *n* and it was concluded they were doubly lysogenic. On the other hand, strain 9684, which was the source of a converting phage identical with that of Bundaberg, had shown free phage on its original plate

and then been picked four times from single colonies; it contained only prophage *a* and had evidently lost the recombinant phage.

The type 80/81 strains used in this study were selected as being representative of isolations made in widely separated parts of the world. The fact that all behaved similarly on lysogenization supports the view that they are closely related, if not identical. This similarity is not, however, proof of a single common origin of all these strains.

Some interesting problems are posed by the appearance of the recombinant phage following infection with the converting phages. So far as we are aware, there is no information on phage crosses and recombination in staphylococcal phages, since experimental systems with suitable genetic markers are at present lacking. On analogy, however, with the coli phages, recombination in the *a* or *n* phages would imply that the 80' prophage is able to enter the mating pool of the infecting phages. The late maturation of the recombinant particles compared with the infecting particles suggests either that this entry occurs at a relatively late stage in the latent period or that, entry having occurred, there is a random chance of the 80' prophage forming a recombinant. Further, there is no definite evidence for or against further replication of the recombinant. Entry into the mating pool also implies that, after infection with the converting phage, there is an alteration in the physical state of the 80' prophage leading to its detachment from its site on the bacterial chromosome. Whether this happens in every type 80/81 coccus infected with a converting phage is unknown but it certainly occurs in all cocci giving a lysogenic response to infection, since all of these lose their 80' prophage.

The term 'converting' phage has been used to describe the phages concerned in this phenomenon. The term is inaccurate when applied to phages *a* or *n*, since the change in the typing pattern of strains of type 80/81 which results from lysogenization with either of these phages is not due to the presence of the phage *per se* but simply to the loss of the defective phage 80'. All the evidence suggests, however, that phage 80' is a converting phage since its presence in the coccus is essential for maintaining the typing pattern of the strain. Its loss, either spontaneously or by prophage substitution, caused an immediate change in the typing pattern. A closer analysis is required of the characteristics of individual recombinants following infection with different phages in order to find out what variations, if any, there are in the genetic contributions made to the recombinants by the infecting phages.

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Pseudomonas maltophilia, an *Alcaligenes*-like Species

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SUMMARY

Pseudomonas maltophilia is frequently encountered in specimens submitted to the clinical laboratory for bacteriological examination. This report describes morphological, physiological and serological attributes of this species. Photomicrographs show the presence of polar multitrichous flagella in stained preparations. These pseudomonads do not produce acid from glucose but readily produce acidity from maltose oxidation. A historical review of the epithet *Alcaligenes bookeri* is presented.

INTRODUCTION

'Bacterium bookeri' NCTC 6572 was isolated in 1943 by J. L. Edwards (Public Health Department, County Bacteriology Laboratory, Stafford, England) from a specimen of pleural fluid and was reported as being non-motile and probably a skin contaminant. It was later found (Hugh, 1953) to have multitrichous flagella. Hugh (1953) recorded the morphological and physiological characteristics of six strains that are now classified as *Pseudomonas maltophilia* including NCTC strain 6572, in a study of *Alcaligenes*. It became apparent that *P. maltophilia* had been unwittingly misidentified as *Alcaligenes faecalis* by Ulrich & Needham (1953); see their strains 249 and 282. An additional 28 strains of *P. maltophilia* have since been isolated from the following sources: river water, well water, raw milk, frozen fish, rabbit and human faeces, blood, pericardial fluid, ascitic fluid, pleural fluid, spinal fluid, oropharyngeal swabs, and contaminated tissue culture. Twenty-six of the above strains have now been restudied and the detailed results are recorded here. An abstract (Hugh & Ryschenkow, 1960) has already been published. The present report serves to amend the previously published description; the name dates from May, 1960 and should be cited as *Pseudomonas maltophilia* Hugh & Ryschenkow, 1960.

METHODS

Morphology. Leifson's (1960) technique was employed to stain flagella.

Physiology. The effects of the bacteria on carbohydrates were determined by the OF (oxidative-fermentative) principle of Hugh & Leifson (1953) and Leifson (1958). The medium used had the following composition: Difco Casitone (pancreatic digest of casein), 5 g.; agar, 3 g.; bromthymol blue (2%, w/v, aqueous solution), 4 ml.; carbohydrate, 10 g.; distilled water, 1000 ml.; final pH 7.1.

Seitz-filtered 10% (w/v) carbohydrate solution was added aseptically to the cool autoclaved (15 min. at 121°) melted basal medium. The medium was then dispensed

aseptically into 13 × 100 mm. sterile tubes to a depth of 50 mm. Ethanol medium was prepared by adding 3 ml. Seitz-filtered absolute ethanol to 97 ml. of the above melted OF basal medium. The ethanol medium was dispensed into sterile 13 × 100 mm. tubes to a depth of 50 mm. and immediately cooled. Ability to grow in an acid medium was studied in yeast extract broth adjusted to pH 4.5 with HCl (Rhodes, 1959) as described by Shimwell, Carr & Rhodes (1960).

Modified Kovacs's reagent (Gadebusch & Gabriel, 1956) was used to detect indole in 1% (w/v) tryptone broth. Simmons's citrate, Christensen's urea, Møller's (1954, 1955) KCN broth, Kligler's iron agar for hydrogen sulphide detection, Kohn's (1953) charcoal gelatin, Carlquist's (1956) test for lysine decarboxylase activity, phenylalanine deaminase activity (Ewing, Davis & Reaves, 1957), the cytochrome oxidase slope test of Ewing & Johnson (1960), and the 2-ketogluconate test of Moore & Pickett (1960) were used. Physiological tests were incubated at 37°; time of incubation and criteria for the tests being positive are given in the references cited and are described by Ewing (1960).

Serology

Preparation of antigen for production of O antisera. Brain heart infusion broth cultures, incubated at 37° for 24 hr., were placed in flowing steam (100°) for 2 hr. and preserved by adding 0.3 ml. of commercial 40% (w/v) formaldehyde to 100 ml. of the broth.

Preparation of antigen for production of H antisera. Actively motile organisms, picked from spreading growth in semi-solid agar Gard (1938) plate incubated at room temperature, were inoculated into brain heart infusion broth. After incubation at 22° for 18–24 hr., the broth was diluted with an equal volume of saline containing 0.6% (v/v) formalin.

Schedule of immunization. Rabbits were given four intravenous injections of H or O antigen at intervals of 4 days. The volume administered was increased each time as follows: 0.5, 1.0, 2.0 and 4.0 ml. The animals were bled on the sixth day after the last injection. Normal serum for control was obtained from each rabbit before immunization. The sera were preserved by the addition of an equal volume of glycerol and stored at 4°.

Preparation of antigens for O and H agglutination. Antigen for O slide-agglutination tests was prepared from an infusion agar slope incubated at 37° for 24 hr. The growth was emulsified in 0.5 ml. saline to form a dense homogeneous suspension. The suspensions were placed in a boiling water bath for 15 min., cooled and tested. Antigen for H tube agglutinations was prepared in the same manner as antigen used for the production of H antisera.

Serological technique. The somatic agglutination of an organism was determined by slide agglutination with a 1/10 dilution of the O antisera. The technique described by Edwards & Ewing (1955) for the somatic agglutination of salmonellas was followed. A twelve tube serial dilution of H antiserum was used to determine flagellar agglutination of the organisms. Tubes were examined for flocculent agglutination after incubation at 45° for 1 hr. followed by 18 hr. at 10°.

RESULTS

Morphology

The organism in peptone broth was a Gram-negative rod about $0.5 \times 1.5 \mu$. Capsule and spore formation was not demonstrable. All the strains were motile and showed a tuft of polar flagella. Plate 1 illustrates the polar multitrichous morphology of *Pseudomonas maltophilia*. The flagella appear to be similar in wavelength and amplitude to the flagella of a typical strain of *P. aeruginosa*.

Physiology

This strictly aerobic organism produced a dense turbidity in peptone broth in 18–24 hr. The strains studied did not produce a distinctive pigment in ordinary media. Smooth glistening growth with an entire margin readily appeared on nutrient agar and blood agar in 24 hr. at 37°. All strains produced grey to white colonies on Leifson's deoxycholate agar. A few strains required 48 hr. to grow out on this medium. Sheep erythrocytes in infusion agar base around well-isolated surface colonies of all 26 strains were not haemolysed. The tube haemolysis test with sheep erythrocytes, as performed in the study of cholera vibrios (Pollitzer, 1959), was negative for all strains. None of the strains grew in peptone broth at pH 4.5.

The following physiological reactions were negative for all 26 strains: acidity from arabinose, glucose, galactose, lactose, mannitol, rhamnose, sucrose, xylose; the indole, methyl red, and Voges-Proskauer tests; urea hydrolysis, nitrate conversion to nitrogen gas, hydrogen sulphide production, phenylalanine deaminase, and 2-ketogluconate production.

The following physiological reactions were positive for all 26 strains: acidity from maltose (acidity was produced in the open tube, but not in the closed tube), motility, gelatin, catalase and lysine decarboxylase. The gelatin test generally became positive before the fourth day. Acidity from maltose generally became evident on the first or second day. Freshly isolated strains of *Pseudomonas maltophilia* inoculated into the basal medium and basal medium containing glucose or other carbohydrates which were not metabolized, produced a strong alkaline reaction at the surface of the medium after 24–48 hr. of incubation. Some old strains which have been kept alive in the laboratory for 8–10 years by periodical transfer in semi-solid medium now produce a very weak acid reaction after prolonged incubation in glucose medium. Some strains produced a positive reaction, while others produced a negative reaction in the tests listed in Table 1.

Antigenic structure

O antigens. Fifteen distinct somatic serotypes were encountered among the 26 strains of *Pseudomonas maltophilia* (Table 2). Organisms of one somatic serotype did not appear to share major antigens with organisms of other serotypes, since cross-reactions between the various O groups were not pronounced. Slow and weak minimal agglutination reactions, which occurred infrequently, were interpreted to be due to minor antigens and were recorded as negative (–) in Table 2. The serum with which the antigen first reacted established the O group to which the organism was assigned. These positive (+) reactions were prompt and strong.

Table 1. *Physiological variability found in 26 Pseudomonas maltophilia strains*

	Number of strains		Time required for the strains to become positive at 37°
	Positive	Negative	
Acidity from fructose (aerobic)	3	23	Positive on the 1st day, after 2nd day positives began to become alkaline
Acidity from mannose (aerobic)	23	3	Generally positive on the 1st or 2nd day
Citrate utilization	6	20	Generally positive on 2nd day
KCN resistance	23	3	Positive on 1st or 2nd day
Nitrate reduction to nitrite	11	15	Test performed after 48 hr. incubation
Cytochrome oxidase slope test	16	10	Test performed after 24 hr. incubation
Acidity from ethanol (aerobic)	1	25	4 days

Living suspensions of *Pseudomonas maltophilia* and suspensions treated with ethanol and heat were also used in the O slide-agglutination test. Of the three different O antigen preparations tested, the boiled suspensions were most suitable. The ethanol treated suspensions and the living suspensions frequently produced a slow and weak agglutination, or no agglutination, with homologous antisera. The information recorded in Table 2 was obtained with boiled suspensions of *P. maltophilia*. Undiluted glycerol-preserved normal control serum did not agglutinate the corresponding organism used for immunization.

Table 2. *O agglutination of Pseudomonas maltophilia with antisera prepared against the corresponding organism*

Antigen	Antiserum														
	560	810-2	294	555	609	601	557	447	558	653-4	788-3	556	109-4	363-4	873-3
560, 430, 559, 661-1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
810-2, 104-2, 245-3	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
294	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
555	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
609, 229, 194-1, 457-1	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
601, 611	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
557, 751-4	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
447	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
558, 483-2	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
653-4	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
788-3	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
556	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
109-4	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
363-4	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
873-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

+ = strong agglutination reaction.

- = no agglutination or weak and slow agglutination.

The 15 *Pseudomonas maltophilia* O antisera did not agglutinate O antigens prepared from 2 strains of each of the following 8 organisms: *Aeromonas hydrophila*, *Alcaligenes faecalis* (peritrichous), *Bordetella bronchiseptica*, *Herellia vaginicola*,

Lophomonas alcaligenes, *Pseudomonas aeruginosa*, *Mima polymorpha*, *Pseudomonas diminuta*. The 16 O antigens of the above organisms were prepared in the same manner as were the *P. maltophilia* O antigens.

There was no evidence of an O antigen common to the following three groups of *Pseudomonas maltophilia*: 12 strains of oropharyngeal origin, 5 strains which apparently were the cause of natural infections, and 2 strains of faecal origin.

Two of the O antigen groups were examined by absorption to confirm that the O antigens within each were serologically similar. The homologous somatic antibodies in antiserum produced by immunization with a boiled suspension of strain 560 were removed by absorption with a live suspension of strain 430 or 559 (see Table 3). The homologous somatic antibodies in antiserum produced by immunization with a boiled suspension of strain 810-2 were removed by absorption with a live suspension of strain 104-2 or 245-3 (see Table 4).

Table 3. *Agglutination following absorption of homologous O antibodies to Pseudomonas maltophilia strain 560 with heterologous organisms*

Living suspension (strain number)	Unabsorbed O antiserum to strain 560	O antiserum to strain 560 absorbed with strain	
		430	559
560	+	—	—
430	+	—	—
559	+	—	—
661-1	+	—	—

Table 4. *Agglutination following absorption of homologous O antibodies to Pseudomonas maltophilia strain 810-2 with heterologous organisms*

Living suspension (strain number)	Unabsorbed O antiserum to strain 810-2	O antiserum to strain 810-2 absorbed with strain	
		104-2	245-3
810-2	+	—	—
104-2	+	—	—
245-3	+	—	—

H antigens. The reciprocal of the homologous titres of each of the six H antisera are tabulated in Table 5. All 26 strains of *Pseudomonas maltophilia* were agglutinated by one or more of the six H antisera. The H antiserum prepared with *P. maltophilia* strain 560 agglutinated 23 of the 26 strains (88%). Eighteen of the strains (69%) appear to have a very similar flagellar antigenic structure. There appear to be at least 4 distinct flagellar antigens.

Glycerol-preserved normal serum in dilutions of 1/20, 1/40 and 1/80 did not agglutinate the corresponding organisms used for immunization.

A fine granular agglutination reaction occurred in some of the H agglutination tests in antiserum dilutions of 1/80 or less. This was best seen with a $\times 10$ hand lens. This phenomenon was interpreted to be due to O antibodies in the H antiserum reacting with the cell bodies of the formalinized flagellar antigen. Such reactions are recorded as negative (—) in Table 5.

Table 5. *Flagellar agglutination of Pseudomonas maltophilia strains with antisera prepared against the corresponding organism*

Suspension (strain no.)	Antisera against strains					
	555	560	810-2	104-2	294	557
	Reciprocal of dilutions					
555	20,480	10,240	2,560	—	—	—
560	20,480	10,240	2,560	—	—	—
447, 556*	5,120 to 20,480	2,560 to 10,240	1,280 to 5,120	—	—	—
810-2, 245-3	10,240	5,120	20,480	20,480	—	—
104-2	—	—	10,240	40,960	—	—
294	—	—	—	—	5,120	—
611	20,480	10,240	2,560	—	5,120	—
229	20,480	2,560	—	—	—	—
430	—	10,240	—	—	—	—
557	—	—	—	—	—	10,240

— = less than 80.

* These organisms and the following were agglutinated over the stated range: 558, 559, 601, 609, 109-4, 194-1, 363-4, 457-1, 483-2, 653-4, 661-1, 751-4, 788-3 and 873-3.

DISCUSSION

The 26 strains of *Pseudomonas maltophilia* examined were morphologically and biochemically much alike, but serologically highly diverse. The following characteristics generally serve to identify the species:

Flagellation	Polar multitrichous
Acid from glucose (aerobic)	No acid
Acid from maltose (aerobic)	Acid
Acid from maltose (anaerobic)	No acid
Acid from mannose (aerobic)	Acid
Citrate utilization	Negative
Potassium cyanide resistance	Positive
Nitrate reduced to nitrogen gas	No gas
Charcoal gelatin	Hydrolysed
Lysine decarboxylase (Carlquist)	Positive
2-Ketogluconate production	Negative

Although no haemolysis was seen around well-isolated colonies, blood agar medium around colonies in the heavily inoculated areas showed a greenish discoloration. This discoloration was interpreted to be the result of excessive accumulation of alkaline metabolic by-products.

The oxidase test for this species of *Pseudomonas* is often positive, delayed and generally difficult to interpret. The positive reactions, obtained with some strains of *Pseudomonas maltophilia*, are generally not as intense as those seen with cholera vibrios and *Aeromonas*. The value of the oxidase test as the sole criterion for the recognition of species of *Pseudomonas* has marked limitations since some species do not produce a positive test and *Vibrio comma* and aeromonads produce a positive test. On the basis of these observations it does not appear judicious to incorporate a positive oxidase test as part of the description of the genus *Pseudomonas*.

The physiological variability found among the 26 strains of *Pseudomonas maltophilia* studied (see Table 1) is interpreted to be variation within the concept of species. No attempt was made to designate physiological varieties of the species in view of the limited number of strains studied and the apparent absence of a practical reason for such division.

Serological reactions did not serve the authors as a useful tool to screen unknown organisms in search of *Pseudomonas maltophilia*. cursory studies on a strain of *P. maltophilia* might result in confusion with *Alcaligenes faecalis*, *Bordetella bronchiseptica* or *Lophomonas alcaligenes*, since all are motile and produce an alkaline reaction in glucose peptone broth. *A. faecalis* and *B. bronchiseptica* have peritrichous flagella. *L. alcaligenes* has lophotrichous flagella and does not attack carbohydrate, hence it differs from *P. maltophilia* which has polar multitrichous flagella and oxidizes maltose and mannose. Clinical bacteriologists frequently fail to differentiate the above organisms because the identification is erroneously thought to be based on a single character or at most only a few characters, and often these are not necessarily the most important for recognition of the taxon. Before the identity of an aerobic, asporogenous Gram-negative, glucose-nonfermenting rod should be regarded as established, the complete morphological and physiological reaction pattern should be determined and compared with the complete pattern of the species in the genus. If the reaction patterns have not been suitably worked out the unknown organism must be compared with recognized authentic type or neotype cultures.

The authors recognize that other species of *Pseudomonas* might be closely related *P. maltophilia*. However, it is difficult to state how other species are related or how they may be precisely differentiated at this stage of our understanding. Ninety-four differently named species of *Pseudomonas* deposited in the American Type Culture Collection were studied; not one of the strains possessed the characteristics of *P. maltophilia*.

Pseudomonas maltophilia 810-2 is designed the type strain. Strains 560, 611, 661-1 and 873-3 may also be useful in future comparative studies. These five strains have been placed in various repositories and have the following corresponding accession numbers:

RH number	Canadian National Research Council number	American Type Culture Collection number
560	—	13636
611	727	13843
661-1	728	—
810-2	729	13637
873-3	730	—

The species name *Alcaligenes bookeri* has been inadvertently assigned to strains included in the above described taxon; hence it would appear appropriate to review the history of this name. Booker (1887) isolated an organism from the faeces of children with diarrhoea. He labelled the organism Bacillus A and described it as actively motile; milk coagulated, alkaline and peptonized; gelatine liquefied. No mention was made of the Gram reaction, carbohydrate reaction or flagellation. Booker (1890) stated that his Bacillus A might have been *Proteus vulgaris*. Ford

(1903) proposed the name 'Bacillus bookeri' for an organism he isolated from the intestine of a child and considered to be like Booker's Bacillus A. He described the organism as: rod shaped; no spores, no effect on glucose, lactose or sucrose; aerobic; yellow or yellow-brown growth on agar slope; litmus milk alkaline and reduced; nitrate to nitrite reduction negative; gelatin, casein and coagulated serum liquefied; indole not produced. There is no statement of Gram reaction or flagellar morphology in Ford's report. It does not seem to us that Ford's 'Bacillus bookeri' was adequately enough described to enable one to recognize the species today with certainty. Levine & Soppeland (1926) proposed the name 'Bacterium bookeri'. Weldin (1927) and Kutscher (1937) did not describe the flagellar morphology of their strains of *Alcaligenes bookeri*. Subsequent literature reviews, such as *Bergey's Manual* (1923, 1957) also used the name *A. bookeri*. *A. bookeri* is described as an organism with the general properties of alcaligenes and specifically with peritrichous flagella and ability to liquefy gelatin; however, the source of this description has not been made evident. It is generally agreed that the type species *Alcaligenes faecalis* is a peritrichous organism. It follows that any other flagellated species included in the genus must also be peritrichous. In view of these comments we consider that *A. bookeri* is a species *incertae sedis* and therefore the epithet *bookeri* should not be applied to *Pseudomonas maltophilia*. In addition, *P. maltophilia* is a polar flagellated organism; hence it is not appropriate to place the taxon in the genus *Alcaligenes*. A review of the literature has not as yet been productive in establishing an earlier epithet for the taxon described in this report.

The specific epithet and noun *maltophilia* is derived from the old Anglo-Saxon word *malt* and the Greek work *philia*. It has the literal word meaning 'malt friend' or 'friend of malt'. The etymology of the specific epithet *P. maltophilia* makes it clear that it is a hybrid epithet. This is a subsidiary point and does not affect the acceptance of the name.

The utilization by bacteria of disaccharides without utilization of either of its constituent monosaccharides has been observed repeatedly. Pelczar & Doetsch (1949) described a *Neisseria* sp. which utilized maltose with acid production while glucose was not utilized. The authors can confirm the existence of such an unnamed *Neisseria* sp. in the oropharyngeal region. *Pseudomonas maltophilia* is yet another example of an organism with this type of physiology.

Liu (1961) concluded that identification of pseudomonads is difficult because of the lack of a biochemical reaction pattern for each species and that identification of species is dependent upon unstable pigment production and unstable pathogenicity. The observations presented in this study of *Pseudomonas maltophilia* do not offer support for these conclusions. The following redefinition of the genus *Pseudomonas* has been found useful: Gram-negative straight or curved rods, polar-flagellated when motile; strict aerobes which do not produce acid from glucose under anaerobic conditions in a glucose peptone medium, glucose may be oxidized to acid end products in the presence of oxygen; indole, methyl red and Voges-Proskauer tests negative; pigments when present are usually water soluble.

During the later stages of preparation of this manuscript in 1961 Dr J. Tannenberg (Genesee Laboratory, Batavia, New York, U.S.A.) informed us that *Pseudomonas maltophilia* strain 1144 was isolated in pure culture at a necropsy from granulomatous lesions in the lung and heart musculature. Bacterial colonies appeared to be present

within small granulomata at the boundary between necrosis and granulomatous cells. Strain 1144 was agglutinated by O antiserum 560. Strains 560, 430, 559 and 661-1 were isolated from spinal fluid, human oviduct, chest fluid and buccal cavity of a normal adult, respectively. Dr J. Tannenberg will amplify these observations and prepare a report later.

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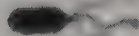
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EXPLANATION OF PLATE

- Fig. 1. *Pseudomonas maltophilia* strain 611 showing a rod with a polar tuft of seven short flagella; $\times 2000$.
- Fig. 2. *P. maltophilia* strain 611 showing a rod with a polar tuft of five flagella; $\times 2000$.
- Fig. 3. *P. maltophilia* strain 611 showing a rod with a polar tuft of four flagella; $\times 2000$.
- Fig. 4. *P. maltophilia* strain 611 undergoing binary fission with one of the two daughters showing a polar tuft of three flagella; $\times 2000$.
- Fig. 5. *P. maltophilia* strain 611 showing a rod with a polar tuft of three flagella; $\times 2000$.
- Fig. 6. *P. maltophilia* strain 611 showing a rod with a polar tuft of two flagella; $\times 2000$.
- Fig. 7. *P. maltophilia* strain 611 undergoing binary fission with both daughters showing a polar tuft of two flagella at the distal ends; $\times 2000$.
- Fig. 8. *P. maltophilia* strain 611 showing a rod with a single long polar flagellum; $\times 2000$.



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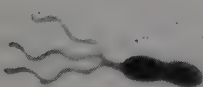
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Taxonomic Relationships of *Xanthomonas uredovorus*

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SUMMARY

The cultural and physiological characteristics of some *Xanthomonas* spp. that are typical of the genus were compared with two atypical species, *Xanthomonas uredovorus* and *X. stewartii*. Electron microscopic studies of *X. uredovorus*, prepared by two methods, show that this organism possesses peritrichous flagella. This fact, together with evidence of the fermentative metabolism of carbohydrates, should exclude this bacterium from the genus *Xanthomonas*. The interpretation of electron micrographs is discussed, and the systematic position of *X. uredovorus* and *X. stewartii*.

INTRODUCTION

From the systematic examination of numerous xanthomonads from culture collections and of fresh isolates from diseased plant material, and from a comparison of these with published descriptions of the genus *Xanthomonas* (Burkholder & Starr, 1948; Dowson, 1957; Dye, 1959) it became obvious that the definition of the genus required clarification. At least two species, *X. uredovorus*, a parasite of the uredia of cereal rusts, and *X. stewartii* the cause of Stewart's disease of sweet corn, were thought to have a number of characteristics which should exclude them from the genus. This study was undertaken in order to compare some physiological characteristics of a selection of seven *Xanthomonas* spp. with *X. stewartii* and *X. uredovorus*, and thus elucidate some fundamental differences between them. An electron microscopic study of *X. uredovorus* was carried out to provide unequivocal evidence of the mode of flagellation. In the original publication (Pon *et al.* 1954) *X. uredovorus* was described as possessing a single polar flagellum, and by this criterion, together with pigmentation, the bacterium was classified in the genus *Xanthomonas*.

METHODS

Source of cultures. The following cultures were obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB, Harpenden, Hertfordshire): *Xanthomonas stewartii* NCPPB 67, NCPPB 449, *X. uredovorus* NCPPB 391, *X. phaseoli* NCPPB 557, *X. campestris* NCPPB 279, *X. vasculorum* NCPPB 795 (from Madagascar), NCPPB 796 (from Mauritius), *X. vesicatoria* NCPPB 701, *X. nigromaculans* f.sp. *zinniae* NCPPB 799, *X. malvacearum* NCPPB 634. Three other cultures of *X. uredovorus* were received from Professor M. P. Starr (University of California); these strains, XU 102, XU 103 and XU 104 have been deposited in the

National Collection of Plant Pathogenic Bacteria and given the accession numbers 800, 801 and 802. Three cultures of *X. malvacearum* from C. Logan (Empire Cotton Growing Corporation, Uganda) and two freshly isolated strains of *X. pruni* from Professor H. H. Thornberry (University of Illinois), were included in the group of strains used for systematic examination.

Staining reactions. Weigert's modification of Gram's method was used. The presence of sudanophilic inclusions in heat-fixed smears of the test bacteria was determined by the method of Burdon (1946), but without clarification of the smears stained with Sudan Black B (G. Gurr Ltd.) by dipping in xylene. After staining with the Sudan stain for 10–15 min. the preparation was washed under a gentle stream of tap water. Smears for Sudan staining were prepared after incubation for 3 and 6 days on glucose peptone agar of the following composition: glucose, 20.0 g.; K_2HPO_4 , 0.5 g.; $MgSO_4 \cdot 7H_2O$, 0.25 g.; peptone (Oxo Ltd.) 5.0 g.; agar (Davis Gelatine Ltd.), 20.0 g.; distilled water 1 l.; pH about 7.2.

Motility. This was determined periodically in a semi-solid medium (3.0 g. agar/l.), using hanging drop preparations.

Oxidation v. fermentation of carbohydrates. The medium of Hugh & Leifson (1953) was modified for use with the weakly oxidative plant pathogenic bacteria, as follows: peptone (Oxoid), 1.0 g.; $NH_4H_2PO_4$, 1.0 g.; $MgSO_4 \cdot 7H_2O$, 0.2 g.; KCl, 0.2 g.; agar, 3.0 g.; bromthymol blue 0.03 g.; distilled water 1 l.; pH about 7.2. Five ml. of a 10% (w/v) solution of glucose was added to 45 ml. quantities of the molten agar base which was dispensed in sterile plugged test tubes (5 × 0.4 inch) to a depth of about 1.5 in. Sterile liquid paraffin (B.P. grade) was used as a seal, following stab inoculation of the test bacteria from agar cultures. Salicin (10.0 g./l) was sterilized with the medium. Inoculated tubes were examined for a period of 14 days.

Lipase. The method of Sierra (1957) was used; the cultures were discarded after incubation for 6 days.

Hydrolysis of soluble starch, gelatin and casein. The following agar medium was dispensed in 45 ml. quantities in 2 oz. bottles: peptone (Oxoid), 5.0 g.; yeast extract (Difco), 3.0 g.; agar, 20.0 g.; distilled water, 1 l.; adjusted to pH 7.2. Five ml. quantities of 4.0% (w/v) gelatin (British Drug Houses Ltd.), 4.0% (w/v) casein (Judex Ltd., light white soluble), or 2.0% (w/v) soluble starch (British Drug Houses Ltd., Analar) were added to the molten agar base from which three plates were poured. Three or four organisms were inoculated to each plate, into the centre of 0.5 cm. diameter cavities made with a surface sterilized cork borer. After incubation for 6 days starch plates were flooded with Gram's iodine solution and gelatin plates with acid mercuric chloride solution (Frazier, 1926). Zones of hydrolysis were recorded.

Aesculin hydrolysis. The liquid and solid media of Sneath (1960) were used and observed for blackening and loss of fluorescence for a period of 14 days.

Production of H_2S and indole. Peptone water supplemented with casein hydrolysate (British Drug Houses Ltd., Laboratory reagent) 1.0 g./l., and L-cysteine hydrochloride, 0.1 g./l. was dispensed in 5 ml. quantities in $\frac{1}{2}$ oz. screw-capped bottles. Lead acetate papers were held over the medium by the screw cap which was kept loose. At 6 days final observations were made for H_2S production, and indole was tested for by addition of ether and Ehrlich's rosindole reagent (Mackie & McCartney, 1960, p. 609). For H_2S production consistent results were only obtained

by using a relatively massive inoculum, i.e. one loopful of growth on glucose peptone agar. From small inocula some *Xanthomonas* spp. gave inconsistent results.

Urease. The method of Christensen (1946) was used, with incubation for 14 days.

Oxidase. Kovacs's method (1956) was used.

Catalase. Loopfuls of agar growth were emulsified in 10 vol. — H_2O_2 on a slide and examined microscopically for evolution of oxygen.

Voges-Proskauer reaction. This was made at 6 days in standard glucose phosphate medium, using Barritt's modification (Mackie & McCartney, 1960).

Salt tolerance. The following medium was dispensed in 10 ml. quantities in 1 oz. screw-cap bottles, and inoculated with two drops of a light suspension of the test organism in distilled water: peptone (Oxoid), 5.0 g.; sucrose (British Drug Houses Ltd., Analar), 5.0 g.; K_2HPO_4 , 0.5 g.; $MgSO_4$, 0.25 g., sodium chloride (Analar), 20.0, 30.0, 40.0 or 50.0 g.; distilled water 1 l.; adjusted to pH 7.2. The culture medium was examined for turbidity during incubation for 14 days.

Nitrite formation from nitrate and nitrite destruction. A medium of the following composition was used: peptone (Oxoid), 10.0 g.; K_2HPO_4 , 5.0 g.; yeast extract (Difco), 1.0 g.; KNO_3 , 1.0 g. or $NaNO_2$, 0.1 g.; agar, 3.0 g.; distilled water, 1 l.; adjusted to pH 7.0; 10 ml. medium per 1 oz. screw-capped bottles. At 5 days tests for nitrite formation or nitrite destruction were made by standard procedures.

Growth temperatures. Two drops of a light suspension of organisms in distilled water were added to glucose peptone agar slopes maintained in a water bath at 40° or 37° for 2 days. Otherwise all cultures were incubated at 28° for the period of test.

Electron microscopy. One culture of *Xanthomonas uredovorus*, strain XU 102, was examined extensively under the electron microscope by one of us (W.H.). Cultures for electron microscopy were grown on nutrient agar slopes and incubated at 20° for 3 days or at 25° for 24 hr. The resultant growth was suspended in distilled water and washed three times by centrifugation. The washed suspensions were then stored overnight at +5°, a procedure which causes partial lysis and consequently causes the cell contents to shrink more readily upon drying. Two methods of preparation, metal-shadowing and phosphotungstic acid 'staining', were used. For the former, the washed suspensions were mounted on formvar films and shadowed with gold-palladium at an angle of 15°. For the phosphotungstic acid preparations (Brenner & Horne, 1959) an equal volume of 2% (w/v) aqueous phosphotungstic acid solution (adjusted to pH 7.4 with KOH) was added to washed suspensions and the resultant mixture mounted on carbon films. The specimens were examined in a Siemens Elmiskop I using the single condensed system with a 200 μ condenser aperture and a 50 μ objective aperture. Electron micrographs were taken at initial magnifications of $\times 8000$ and $\times 10,000$ on Ilford N 50 plates.

RESULTS

All the bacteria examined were unequivocally Gram-negative in young (16 hr.) cultures and did not form prominent polar or central, refractile, sudanophilic inclusions on glucose peptone agar after incubation for 3 or 6 days. This is in accordance with previous observations (Hayward, 1960). With the known exception of *Xanthomonas albilineans*, bacteria of the genus *Xanthomonas* do not form sudano-

philic inclusions in culture on a medium containing utilizable carbohydrate, as do many pseudomonads (Morris & Roberts, 1959).

There were differences between the cultural characteristics of the *Xanthomonas* spp. on the one hand and the cultures of *X. stewartii* and *X. uredovorius* on the other. The latter did not produce the raised, convex, shiny, slimy, mucoid colonies which are typical of the majority of *Xanthomonas* spp. grown on an agar medium containing glucose or sucrose. The exception in this study was the Madagascar strain of *X. vasculorum* which was non-mucoid, in common with several other strains of this species. Elrod & Braun (1947) noted the increase in degree of mucoidness with serial transfer on media rich in carbohydrate, an increase we have noted. This increase in mucoidness was observed in some strains which were non-mucoid on first isolation. All the bacteria examined were yellow in the mass on agar.

The cultures examined were catalase positive. The Kovacs oxidase test did not give unequivocal results on different media and after different periods of incubation: on Difco nutrient agar cultures at 2 days all the *Xanthomonas* spp. were positive, i.e. they gave a strong colour reaction in less than 30 sec., while the cultures of *X. uredovorius* and *X. stewartii* were negative at 30 sec. However, on a medium

Table 1. *Characters of some Xanthomonas spp., Xanthomonas stewartii and Xanthomonas uredovorius*

Characteristic	<i>Xanthomonas</i> spp. (7 species, 12 strains)	<i>X. stewartii</i> (2 strains) NCPB 449 NCPB 67	<i>X. uredovorius</i> (4 strains)
Motility	+	—	+
Metabolism of glucose	Oxidative (3–6 days)	Fermentative, anaerogenic (24–48 hr.)	Fermentative, anaerogenic (12–24 hr.)
Metabolism of salicin	—	—	Fermentative, anaerogenic
Hydrolysis of 'Tween 80' (lipase)	+	—	+
Soluble starch hydrolysis	+($\frac{9}{11}$)	—	—
Gelatin hydrolysis	+	—	+
Casein hydrolysis	+	—	Weak +
Aesculin hydrolysis	+	—	+
H ₂ S from cysteine	+	—	—
Nitrite from nitrate	—	—	+
Nitrite destruction	—	—	—
Tolerance of sodium chloride	2–3 %	5 %	5 %
Urease	—	—	—
Voges-Proskauer reaction	—	— (449), + (67)	—
Indole production	—	—	+
Growth at 37°	+	+	+
Growth at 40°	—	—	—

— = Negative reaction, + = positive reaction.

Soluble starch hydrolysis: for *Xanthomonas* spp. zone diameter 36.0–56.0 mm. The two cultures of *X. pruni* did not hydrolyse starch, the Madagascar strain of *X. vasculorum* did not produce a clearly defined zone of hydrolysis but showed a trace of activity.

Casein hydrolysis: for *Xanthomonas* spp. zone diameter 17.0–46.0 mm. The cultures of *X. uredovorius* did not produce a clearly defined zone of hydrolysis but a decrease in the opacity of the medium in a zone 10.0 mm. diameter.

Gelatin hydrolysis: for *Xanthomonas* spp. zone diameter 20.0–46.0 mm., for the cultures of *X. uredovorius* c. 15.0 to c. 23.0 mm.

containing 2% (w/v) glucose or glycerol the Kovacs reaction given by some of the *Xanthomonas* spp. was retarded and often indistinguishable from the reaction given by *X. uredovorus* and *X. stewartii*. On a rich tomato juice agar medium the cultures of *X. uredovorus* and *X. stewartii* gave a positive oxidase reaction.

In the modified Hugh & Leifson (1953) technique *Xanthomonas* spp. produced an indicator change in the top 1 cm. of medium after incubation for 3–6 days, whereas the cultures of *X. uredovorus* produced an indicator change throughout the depth of the medium in 12–24 hr. and *X. stewartii* in 24–48 hr. Other characters are given in Table 1.

Electron microscopy of Xanthomonas uredovorus strain XU 102.

Shadow-cast specimens showed that the organisms usually possess one or two lateral flagella (Pl. 1, figs. 1, 2, 3); occasionally more flagella were seen (Pl. 1, figs. 4, 5 and 6). No organisms with polar flagella were seen. The phosphotungstic acid preparations were most interesting as these illustrate the difficulties encountered in the interpretation of flagella-preparations. Plate 2, fig. 7, is easy to interpret and clearly shows the lateral insertion of the flagellum. However, were this organism to be rotated on its own axis through 90° the flagellum could appear to be polar in origin, particularly in shadow-cast specimens. Plate 3, fig. 8, illustrates the true path of the flagellum and the origin of the flagellum in an organism in exactly this position.

Plate 4, fig. 9, shows a shadow-cast specimen in which the true path of the flagellum can be traced. Partial lysis and the consequent amount of shrinkage of cell contents allows the true (lateral) position of origin of the flagellum to be clearly defined.

DISCUSSION

The electron micrographs indicate that the original authors (Pon *et al.* 1954) were in error in their interpretation of their electron micrographs. Critical examination of their published micrograph (Pon *et al.* 1954) reveals the possible source of this error. The complete organism shown in the micrograph is full of cell material, it is metal-shadowed and there is a considerable amount of debris near the pole. The true path of the flagellum is probably obliterated by the metal shadowing (cf. Pl. 3, fig. 8; Pl. 4, fig. 9). In addition the Pon *et al.* micrograph includes part of another cell in which the origin of the flagellum is undoubtedly lateral. Attention has already been drawn to the difficulties of interpretation of flagella stains when using the optical microscope (Hodgkiss, 1961). It is apparent from the present results that electron-micrographs of bacterial flagella require equally careful interpretation. As an illustration, an overexposed negative or an overexposed and/or overdeveloped print of Pl. 3, fig. 8 could show a 'polar' flagellum.

The occurrence of mixed polar and peritrichous forms in pure cultures of organisms as described by Leifson & Hugh (1953), Sneath (1956) and Sreenivasan & Venkataraman (1956) merits discussion. Careful electron microscopic studies at Torrey Research Station of various organisms have so far failed to yield similar results. It would appear that the interpretation of results requires special care in optical-microscopic examinations of flagella stains when detail such as that seen in Pls. 2, 3, 4 cannot possibly be resolved.

Together with the evidence provided by electron microscopy, the results of the biochemical tests show that *Xanthomonas uredovorus* has no place in the Pseudo-

monadaceae, but should be included in the family Enterobacteriaceae. *X. uredovorus* is not a plant parasite, but a parasite of the fructifications of a fungal pathogen and the bacterial genus *Erwinia* is so defined in the 7th edition (1957) of *Bergey's Manual* to include only plant pathogens. However, *X. uredovorus* is closely related to the anaerogenic *Erwinia* spp., to which genus we propose that this bacterium be transferred.

Misgivings about the classification of *Xanthomonas uredovorus* were expressed by Lovrekovich & Klement (1960) in a study of the tolerance of triphenyltetrazolium chloride by organisms of several genera of bacterial plant pathogens. In the genus *Xanthomonas*, *X. uredovorus* strains, and also *X. stewartii*, were markedly more tolerant than the other species tested.

Xanthomonas uredovorus is related to *Erwinia lathyri* (Manns & Taubenhaus) Holland as described by Graham (1958), who summarized the principal characters of this bacterium as follows: coliform (i.e. fermentative) metabolism of carbohydrates, without gas formation, peritrichous flagellation, positive gelatin liquefaction, Voges-Proskauer and nitrate reduction both usually positive, formation of a yellowish pigment on many agar media. Although discredited as a plant pathogen (Graham, 1958), *E. lathyri* or its close relatives are regularly isolated from moribund plant material. They occur as saprophytes on plants and plant debris and in the soil, which is also characteristic of *Xanthomonas uredovorus* according to Pon *et al.* (1954). *E. lathyri* grows more rapidly on isolation media than xanthomonads, and consequently has often been implicated, mistakenly, as the cause of plant disease. In differentiating the Gram-negative bacteria on plant material the technique of Hugh & Leifson (1953) has proved invaluable for screening a large number of bacteria, and in avoiding the confusion between *E. lathyri* and *Xanthomonas* spp.

The definition of the genus *Xanthomonas* (Dowson, 1957; Dye, 1959) should be modified to include only bacteria with an oxidative metabolism of glucose, and thus exclude organisms such as *X. stewartii* and *X. uredovorus* which are clearly distinct from *Xanthomonas* on other grounds (see Table 1). *X. stewartii*, in addition to having a fermentative metabolism of carbohydrates, is non-motile, has a high salt tolerance (Burkholder & Starr, 1948), does not hydrolyse aesculin, potato starch or gelatin, and does not produce hydrogen sulphide in a medium containing cysteine. However, the systematic position of *X. stewartii* is obscure, and without detailed comparative study with other yellow Gram-negative bacteria, for example, of the genus *Flavobacterium*, it is not yet possible to propose a change in the classification of this organism. It has long been recognized as an atypical xanthomonad (Burkholder & Starr, 1948), and may be a degenerate member of the Enterobacteriaceae.

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EXPLANATION OF PLATES

All preparations were of cultures of *Xanthomonas uredovorus*, strain XU 102 grown on nutrient agar at 20° for 3 days.

The scale marked on figures = 1.0 μ .

PLATE 1

- Fig. 1. Gold palladium shadow at 15°. $\times 16,000$. Neg. no. 454.
Fig. 2. Gold palladium shadow at 15°. $\times 25,000$. Neg. no. 452.
Fig. 3. Gold palladium shadow at 15°. $\times 16,000$. Neg. no. 481.
Fig. 4. Gold palladium shadow at 15°. $\times 20,000$. Neg. no. 458.
Fig. 5. Gold palladium shadow at 15°. $\times 24,000$. Neg. no. 456.
Fig. 6. Phosphotungstic acid preparation. $\times 32,000$. Neg. no. 472.

PLATE 2

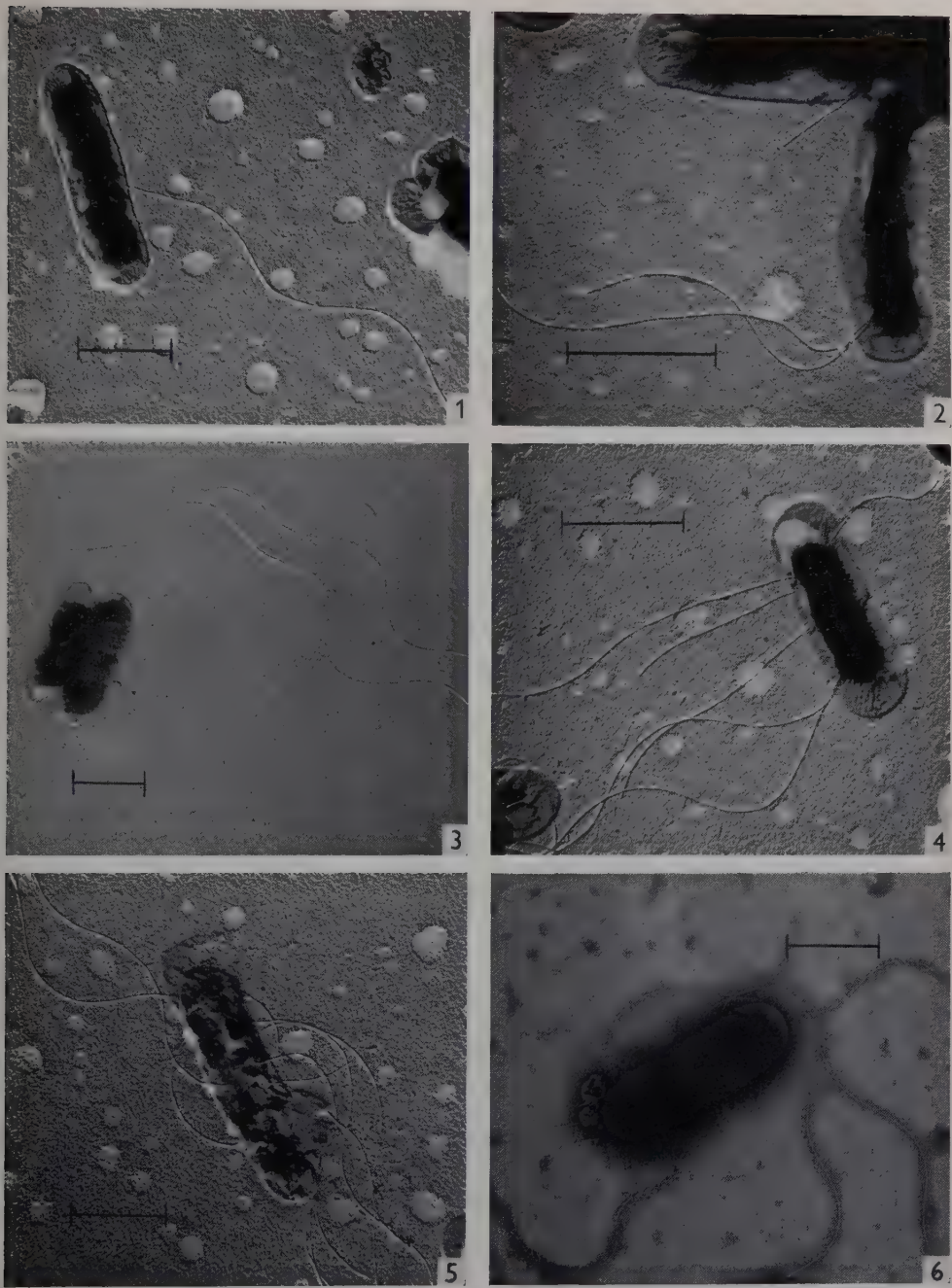
- Fig. 7. Phosphotungstic acid preparation. $\times 32,000$. Neg. no. 472.

PLATE 3

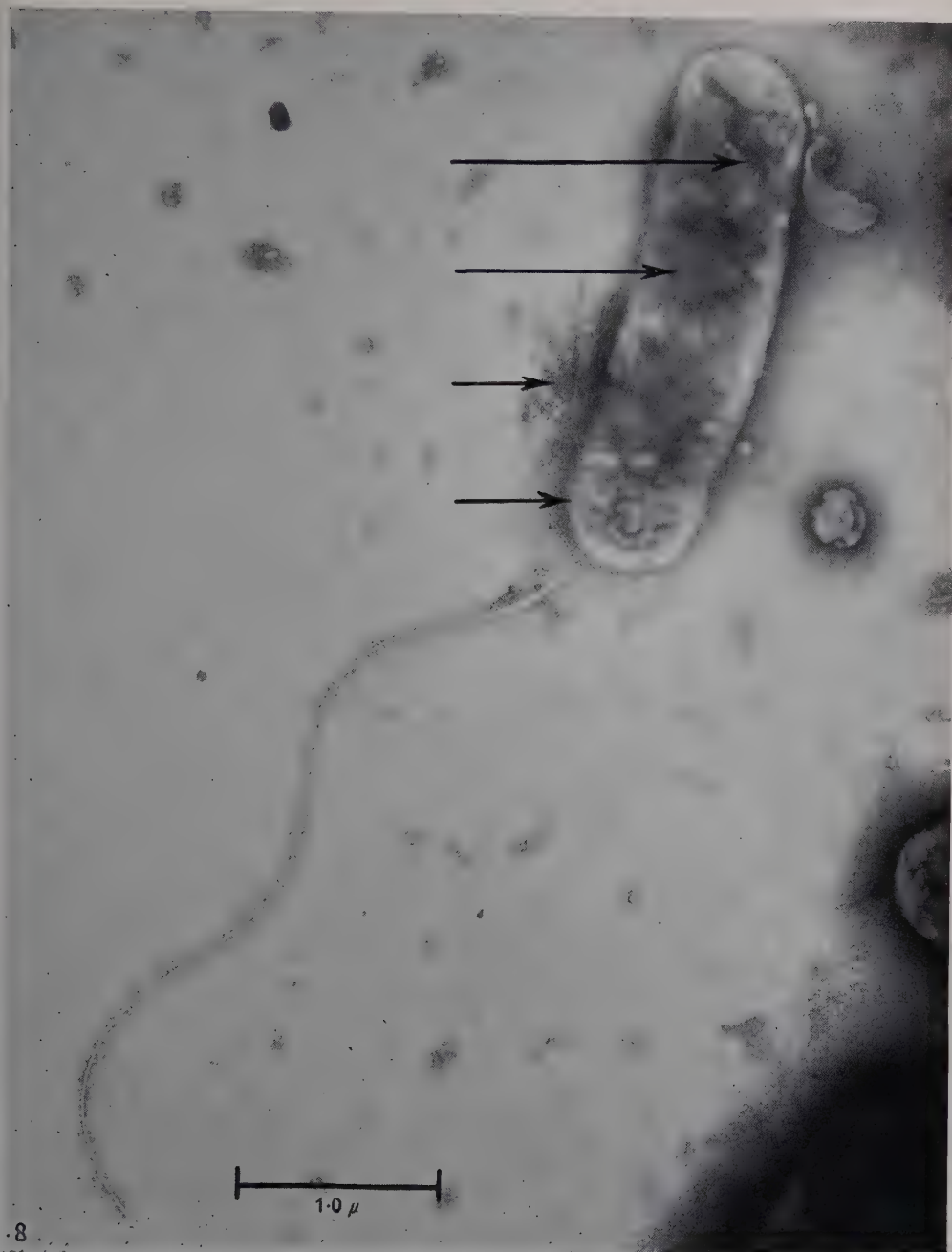
- Fig. 8. Phosphotungstic acid preparation. $\times 32,000$. Neg. no. 478.

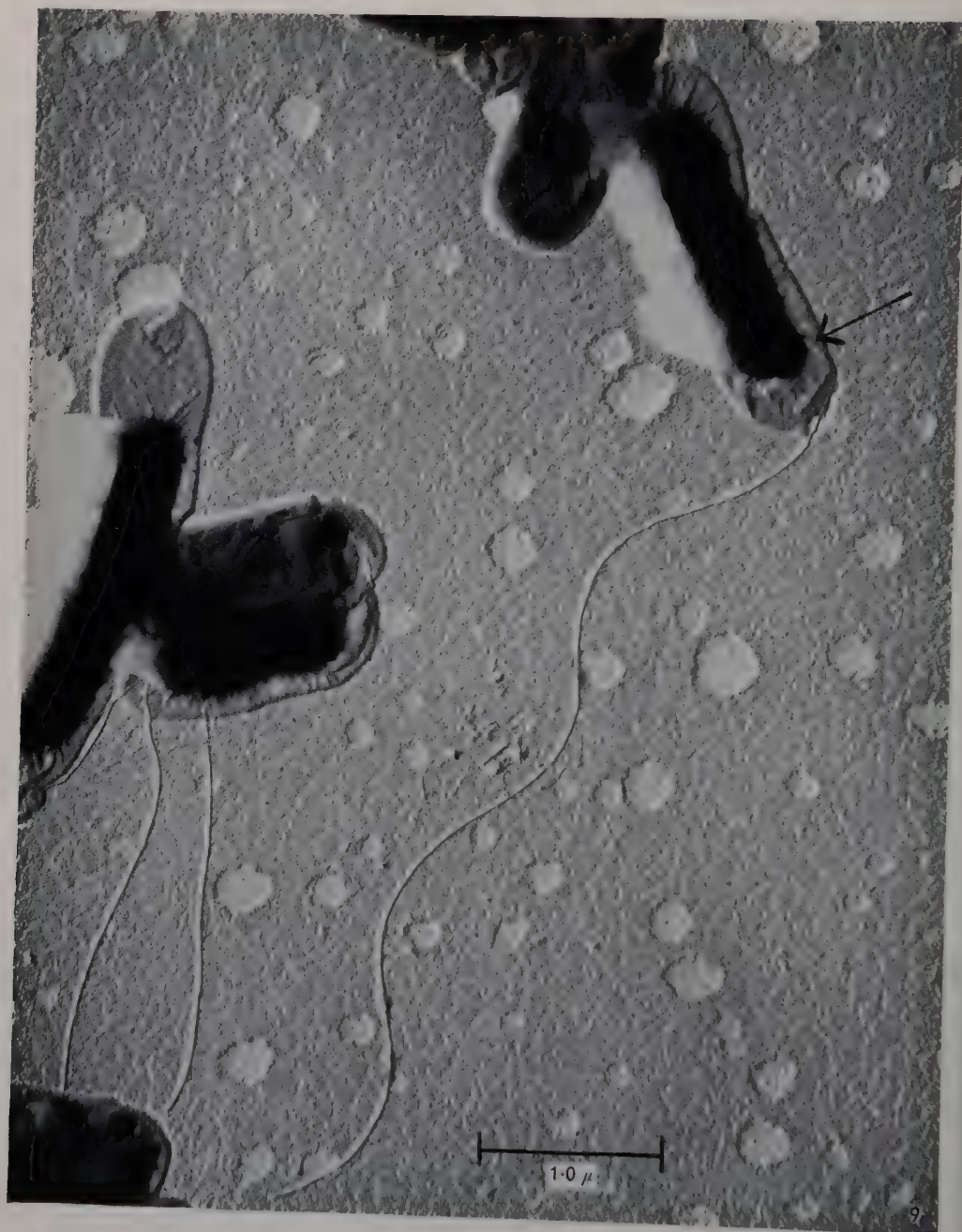
PLATE 4

- Fig. 9. Gold palladium shadow at 15°. $\times 32,000$. Neg. no. 482.









Metschnikowiella zobellii sp.nov. and *M. krissii* sp.nov., two Yeasts from the Pacific Ocean Pathogenic for *Daphnia magna*

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SUMMARY

Metschnikowiella zobellii sp.nov. and *M. krissii* sp.nov. are described and Latin diagnoses given. Both species form on V8 agar (Wickerham, 1951) club-shaped asci containing a single needle-shaped ascospore and are capable of parasitizing *Daphnia magna* under experimental conditions. *M. zobellii* differs from *M. krissii* by its capacity to ferment glucose and to assimilate galactose, L-sorbose, D-xylose, D-glucosamine, adonitol, D-sorbitol and pyruvate. In infected daphnias the asci and cells of both yeasts resemble Metschnikoff's drawings of *M. bicuspidata*. As cultures of *M. bicuspidata* do not exist and its physiological properties are unknown, the possible identity of either *M. zobellii* or *M. krissii* with *M. bicuspidata* cannot be verified and *M. bicuspidata* (Metschnikoff 1884) Genkel 1913 is therefore considered a *nomen dubium*. Both yeasts were repeatedly isolated from marine substrata on and off the coast of La Jolla, California, U.S.A. Minimum numbers of viable organisms in positive samples varied for *M. zobellii* as follows: sea water, 2-58/100 ml.; fish gut contents (of *Atherinopsis affinis littoralis* and *Trachurus symmetricus*), 25-5,730/ml.; surface of Giant Kelp (*Macrocystis pyrifera*), 520-39,200/g. *M. krissii* was isolated only from sea water, 1-57/100 ml.

INTRODUCTION

Metschnikoff (1884) described as *Monospora bicuspidata* a yeast-like organism, parasitic in the body cavity of the fresh-water crustacean *Daphnia magna*. Keilin (1920) summarized Metschnikoff's observations as follows: 'when the cavity of the hosts is entirely invaded by the parasites, these grow in size, become elongated, and form club- or sausage-shaped asci in each of which is developed a single needle-like spore having both ends pointed. When the parasitized host dies, it is filled with ripe spores, and healthy daphnias, which feed on the detritus of their dead and diseased fellows, become infected by ingesting the asci. The latter, when they enter the host's alimentary canal, set free the needle-shaped spores which perforate the gut wall and penetrate in the body cavity, where they germinate laterally, thus starting the new infection'. It was in this material that Metschnikoff made his early observations on the important phenomenon to which he gave the name of phagocytosis.

According to Kudryavtsev (1954), the generic name *Monospora* was changed by Kamensky (1899) to *Metschnikowia* because *Monospora* had been in use, prior to Metschnikoff, for a genus of Algae. Genkel (1913) found out that *Metschnikowia* had also been in previous use and proposed the name *Metschnikowiella*. Keilin (1920),

unaware of this renaming, introduced the name *Monosporella* and described as *Monosporella unicuspidata* a second species, observed by him in the body cavity of larvae of the peratogonid fly *Dasyhelea obscura*. Kudryavtsev (1954) renamed it *Metschnikowiella unicuspidata*.

Monosporella unicuspidata, as described by Keilin (1920), differs from *Monosporella bicuspidata* by the morphology of its spores: pointed at one end and truncated at the other in the former species, pointed at both ends in the latter. According to Keilin (1920) a similar yeast was observed by Bütschli in the coelom of a free-living nematode, *Tylenchus pellicidus*. Mesnil & Caullery (1911) found yeast-like fungi of elongated shape, in a polychaete worm *Potamilla torelli*, which they considered related to *Monospora*, although they did not succeed in finding spores. Also in a pelagic copepod *Acartia* they observed what they considered to be a similar yeast. None of these yeasts has been cultivated by their authors or by others. Recently one of us isolated from various marine substrata on and off the coast of La Jolla, California, U.S.A., a number of yeasts, which, based on their fermentative and assimilative properties, belong to two distinct species, both morphologically similar with *Monosporella bicuspidata*. We propose for these two species the names *Metschnikowiella zobellii* and *M. krissii* (in honour of Professor Dr C. E. ZoBell and Professor Dr A. E. Kriss, pioneering marine microbiologists, based at La Jolla, California, U.S.A. and Moscow, U.S.S.R., respectively).

METHODS

Isolation medium. The isolation broth had the following composition (% w/v): glucose, 2; peptone (Difco), 1; yeast extract (Difco), 0.5; filtered sea water; isolation agar had 2% (w/v) agar added. To discourage bacterial growth the medium was adjusted to pH 4.5 with lactic acid.

Isolations from sea water. Subsurface samples were taken at the end of the pier of the Scripps Institution of Oceanography, University of California, La Jolla, California, U.S.A.; 100 ml. samples were run through 'Millipore' filters (Millipore Filter Corp.) of the following specifications: HA, pore size $0.45\ \mu$; disk diameter 47 mm.; white; plain. Each filter was then placed on top of plated isolation medium.

The plates were incubated at a temperature between 18° and 20°. After 3–5 days yeast colonies appeared on the filters. These were subcultured to the periphery of the plates. The subcultured colonies were distinguished by macroscopic and microscopic morphology. The numbers of each type were recorded and representatives subcultured to slopes of isolation medium for later identification.

Isolations from fish gut contents. Fish of the species *Atherinopsis affinis littoralis* Ayres ('Topsmelt') and *Trachurus symmetricus* Ayres ('Pacific Jack Mackerel') were caught with unbaited hooks off the coast of La Jolla, California. The gut contents of each specimen were suspended in twice their volume of filter-sterilized sea-water and 0.2 ml. amounts of the suspensions were spread with a glass rod on the surface of isolation medium plates. The plates were incubated at 18°–20°, the numbers of yeast colonies recorded according to macroscopic and microscopic morphology. Representative colonies of each type were subcultured for later identification.

Isolations from Giant Kelp. Huge heaps of Giant Kelp (*Macrocystis pyrifera*) are washed ashore on the beaches of Southern California. Pieces of stems and leaves

were cut from the subsurface of such heaps, placed in sterile Erlenmeyer flasks and weighed. After the addition of four times the weight of filter-sterilized sea water, the flasks were shaken for 50 min. Serial dilutions of the wash water were then plated on isolation medium and the plates, treated in the same way as the gut-contents plates.

Identification of the yeast isolates. The methods described by Lodder & Kreger-van Rij (1952), Wickerham (1951) and Van Uden & Farinha (1958), were used. Only isolates belonging to the *Metschnikowiella* genus are considered in this paper.

Experimental pathogenicity. Yeast-free pond water was placed in 50 ml. amounts in wide-mouthed jars together with twenty adult embryo-bearing females of *Daphnia magna* from a yeast-free laboratory population. Duplicate jars were inoculated with a loopful of growth of a 10-day sporulating culture of either *Metschnikowiella zobellii* or *M. krissii*; one set was left uninoculated as a control. The jars were incubated at 20° in a water bath and inspected daily for the presence of dead daphnias. The latter were examined microscopically in unstained preparations and used for making cultures.

RESULTS

A total of twenty-nine isolates of *Metschnikowiella zobellii* and six isolates of *M. krissii* were obtained. Their abundance in the various substrata examined can be seen from Table 1. As the possibility of seasonal fluctuations of the *Metschnikowiella* populations has not been excluded, the dates of collection are given.

Table 1. *Minimum numbers of viable cells of Metschnikowiella spp. yeasts in marine substrata*

Date of collection (1960)	In 100 ml. seawater		M. zobellii in 1 ml. fishgut contents of		M. zobellii on the surface of 1 g. Giant Kelp (<i>Macrocystis pyrifera</i>)
	M. zobellii	M. krissii	<i>Atherinopsis affinis littoralis</i>	<i>Trachurus symmetricus</i>	
12. ii.	8	1	.	.	12,600
19. ii.	28	4	.	.	23,000
21. ii.	2	0	.	.	760
3. iii.	2	0	.	.	5,720
8. iii.	0	14	2,100	0	.
11. iii.	58	0	275	.	.
14. iii.	16	0	.	.	.
15. iii.	0	25	520	0	.
23. iii.	15	30	.	1,950	0
29. iii.	4	0	.	.	.
30. iii.	3	57	.	.	.
1. iv.	.	.	.	5,730	39,200
11. iv.	42	0	0	.	.
9. v.	17	0	85	.	.
10. v.	47	0	930	.	520
11. v.	22	0	.	25	29,600
13. v.	15	0	.	.	0

0 = no yeast present in sample.

. = no sample taken.

Metschnikowiella zobellii sp.nov.

In medio liquido cum dextroso et peptono et extracto levedinis cellulae rotundae et ovoideae, $(6-11) \times (7-11) \mu$, singulares, binae aut catenatae; pellicula tenuis formatur. In agaro peptonato cum dextroso et extracto levedinis cultura flavalbida, mollis, subnitida, subreticulata. Pseudomycelium primitivum formatur. Asci in agaro V8 clavati, $18-20 \mu$; asci in *Daphnia magna* elongato-subclavati, $30-45 \mu$. Ascosporae acuiiformes, bicuspidatae, ad 1 in asco; in agaro V8, $15-18 \mu$, in *Daphnia magna*, $28-43 \mu$. Dextrosum fermentatur at non galactosum, saccharum, maltosum, raffinose, trehalosum et inulinum. Dextrosum, galactosum, L-sorbose, maltosum, saccharum, cellobiosum, trehalosum, meleucitosum, D-xylose, D-glucose-aminum, alcohol aethylicum, glycerinum, adonitolum, D-mannitolum, D-sorbitolum, alpha-methyl-glucosidum, salicinum, arbutinum, acidum pyruvicum et acidum succinicum assimilantur at non nitrates kalici. Necessaria ad fortem crescentiam sunt vitamina externa: biotinum et thiaminum. Temperatura maxima crescentiae $34^{\circ}-35^{\circ}$.

Morphology. In isolation broth after 48 hr. at 25° cells are round and oval, $(6-11) \times (7-11) \mu$, single, in pairs and in small groups. A thin pellicle is formed. The streak culture on isolation agar after 30 days at 25° is yellowish white, soft, dull-glistening and slightly reticulated. A primitive pseudomycelium is formed.

Sporulation. Asci are formed on Henrici's vegetable juice medium (V8 medium, Wickerham, 1951). No sporulation has been observed to occur on isolation agar, malt agar and corn-meal agar. The asci are club-shaped, $18-21 \mu$ long and contain a single, needle-shaped ascospore, pointed at both ends and $15-18 \mu$ long (Fig. 1).

Fermentation, growth in the absence of single vitamins at 25° and assimilation (see Table 2).

Maximum temperature still permitting growth: $34^{\circ}-35^{\circ}$.

Experimental pathogenicity for Daphnia magna. During the 21 days of observation daphnia populations in the control jars showed no signs of disease and produced a numerous offspring. In the jars inoculated with *Metschnikowiella zobellii*, dead daphnias began to appear after 3-5 days and the entire population had died after 10-14 days. The dead specimens were densely filled with yeasts; on subculture the dead daphnias produced only *M. zobellii*, no other yeasts. The morphology of *M. zobellii* in the daphnias showed a striking similarity with Metschnikoff's drawings of *M. bicuspidata*, as reproduced by Keilin (1920) and Kudryavtsev (1954). The asci were elongated or slightly clavate, $30-45 \mu$ long, and contained a single needle-shaped ascospore, $28-43 \mu$ long. The vegetative cells were more slender than the cells on artificial media (Fig. 2).

Type strain. An isolate from sea water has been designated as the type strain of *Metschnikowiella zobellii*. It is maintained in this laboratory with the number 2892. Subcultures have been deposited with the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, Netherlands and the Fermentation Division of the Northern Utilization Research and Development Division, Peoria, Illinois, U.S.A.

Metschnikowiella krissii sp.nov.

In medio liquido cum dextroso et peptono et extracto levedinis cellulae rotundae et ovoideae, $(4.5-6) \times (6-11) \mu$ et longovoideae, $(4.5-6) \times (11-13) \mu$, singulares, binae

aut catenatae. In agar peptonato cum dextroso et extracto levedinis cultura flavalbida, mollis, subnitida, subpunctata. Pseudomycelium primitivum formatur. Asci in agar V8 clavati, 18–24 μ ; asci in *Daphnia magna* elongato-subclavati vel elongato-angulares, 35–40 μ . Ascospores acuiiformes, bicuspidatae, ad 1 in asco; in agar V8, 15–21 μ ; in *Daphnia magna* 32–38 μ . Non fermentat. Dextrosus, maltosus, saccharus, cellobiosus, trehalosus, meleucosus, alcohol aethylicus, glycerinus, D-mannitolus, alpha-methyl-glucosidus, salicinus, arbutinus et acidum succinicum assimilantur, ad non nitras kalicus. Necessaria ad fortem crescentiam sunt vitamina externa: biotinus et thiaminus. Temperatura maxima crescentiae 34–35°.



Fig. 1

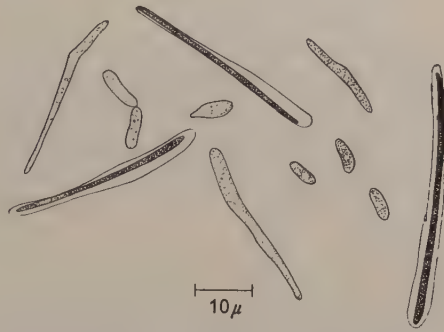


Fig. 2

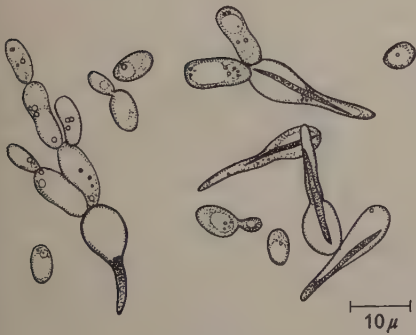


Fig. 3

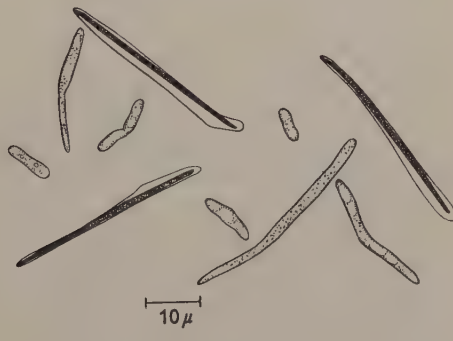


Fig. 4

Fig. 1. *Metschnikowiella zobellii*. Asci and vegetative cells on V8 medium. Camera lucida drawing.

Fig. 2. *Metschnikowiella zobellii*. Asci and vegetative cells in parasitized *Daphnia magna*. Camera lucida drawing.

Fig. 3. *Metschnikowiella krissii*. Asci and vegetative cells on V8 medium. Camera lucida drawing.

Fig. 4. *Metschnikowiella krissii*. Asci and vegetative cells in parasitized *Daphnia magna*. Camera lucida drawing.

Morphology. In isolation broth after 48 hr. at 25° cells are round and oval, (4.5–6) \times (6–11) μ , and long oval, (4.5–6) \times (11–13) μ , single, in pairs and small groups. A thin pellicle may form after prolonged incubation. The streak culture on

isolation agar after 80 days at 25° is yellowish white, soft, dull-glistening and slightly pointed. A primitive pseudomycelium is formed.

Sporulation. Asci are formed abundantly on V8 medium (Wickerham, 1951). Sporulation has not been observed on isolation agar, malt agar and corn meal agar. The asci are club-shaped, 18–24 μ long and contain a single, needle-shaped ascospore, pointed, at both ends and 15–21 μ long (Fig. 8).

Fermentation, growth in the absence of single vitamins at 25° and assimilation (see Table 3).

Maximum temperature still permitting growth: 34°–35°.

Experimental pathogenicity for Daphnia magna. *Metschnikowiella krissii* behaved in the same way as *M. zobellii*. Its morphology in *Daphnia magna* can be seen in Fig. 4. Many asci were slightly angular.

Type strain. An isolate from sea water has been designated as the type strain of *M. krissii*. It is maintained in this laboratory with number 2895. Subcultures have been deposited with the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, Netherlands, and the Fermentation Division of the Northern Utilization Research and Development Division, Peoria, Illinois, U.S.A.

DISCUSSION

Taxonomy. As *Metschnikowiella bicuspidata* and *M. unicuspidata* have not been cultivated in the laboratory, their physiological properties are unknown and no cultures are available for comparative studies. As yeast species identification is largely based on physiological properties, an eventual re-identification of either species would probably imply an arbitrary decision. Their known properties, as described by Metschnikoff and Keilin, are however unique among the yeasts and characterize the genus *Metschnikowiella*: (1) asci containing a single needle-shaped ascospore; (2) pathogenicity for arthropods. We feel justified therefore to place *M. zobellii* and *M. krissii* in *Metschnikowiella*. An emended description of the genus *Metschnikowiella*, a discussion of its possible phylogenetic relationships and the designation of a type species is better postponed, we feel, till the life cycles of *M. zobellii* and *M. krissii* have been worked out. Both *M. zobellii* and *M. krissii* are morphologically similar to *M. bicuspidata* and both are capable, like *M. bicuspidata*, of parasitizing *Daphnia magna*. As far as Metschnikoff's description of *M. bicuspidata* goes, our two yeasts would have to be identified with Metschnikoff's organism. This, however, is impossible since *M. zobellii* and *M. krissii* belong to distinct species. *M. zobellii* differs from *M. krissii* by its capacity to ferment glucose and to use galactose, L-sorbose, D-xylose, D-glucosamine, adonitol, D-sorbitol, and pyruvate. This situation exemplifies that Metschnikoff's description of *M. bicuspidata* is not sufficiently complete to allow its re-identification. As, furthermore, no cultures of *M. bicuspidata* are available to permit an emended description, we have to consider the name *Metschnikowiella bicuspidata* (Metschnikoff, 1884) Genkel, 1913 as a *nomen dubium*.

Ecology. *Metschnikowiella zobellii* and *M. krissii* were repeatedly isolated from sea water and *M. zobellii* also from Giant Kelp and fish gut (Table 1). This shows that both species, though capable of infecting and killing *Daphnia magna*, are facultative, rather than obligate parasites. The numbers of viable *M. zobellii* on the surface of Giant Kelp were very high as compared with the numbers found in sea water. In

most heaps of Giant Kelp from which *M. zobellii* was isolated, slight signs (smell, consistency, temperature) of beginning microbial decomposition were noted. Possibly the breakdown of algal polysaccharides by bacterial action provides certain simple carbohydrates, easily used as a carbon source by *M. zobellii*. The inability of *M. krissii* to assimilate galactose and other carbohydrates may possibly explain why this species was not found in the kelp heaps. The numbers of *M. zobellii* were much higher in fish gut than in sea water. This suggests that *M. zobellii* either multiplies in fish gut more actively than in sea water or is associated with some of the marine organisms on which the fish feed. Both *M. zobellii* and *M. krissii* are capable of parasitizing *Daphnia magna* under experimental conditions. *Daphnia magna*, however, is a fresh-water crustacean, whereas *M. zobellii* and *M. krissii* seem well adapted to marine environments. Whether these species may, under natural or experimental conditions, parasitize marine crustaceans or other marine animals is an open question.

The isolation work was done during a stay of one author (N. van U.) at the Microbiology Laboratories, Scripps Institution of Oceanography, University of California, La Jolla, California, U.S.A. To Professor Dr C. E. ZoBell and his associates Dr G. E. Jones, Mr H. L. Scotten, Mrs Jean S. ZoBell and Miss Susan Wright warm thanks are expressed for their hospitality and help.

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Isolation, Identification and Growth of some Soil Hyphomycetes and Yeast-Like Fungi which Utilize Aromatic Compounds Related to Lignin

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SUMMARY

Fungi were isolated from soil under several vegetational types by an enrichment technique with vanillin or *p*-hydroxybenzaldehyde as sole source of carbon. Although similar morphologically, the isolates obtained are classified in two separate groups, yeasts and hyphomycetes. A study was made of the growth in pure culture of representative species, namely, *Pullularia pullulans*, *Margarinomyces heteromorpha* and *M. mutabilis* on several aromatic compounds related to lignin.

INTRODUCTION

In previous studies concerning the decomposition of lignin by soil fungi (Henderson & Farmer, 1955) a number of isolates were obtained from soil by means of the dilution-plate method. Apart from incorporating tannic acid in the medium used, in the hope that lignin-decomposing fungi might be indicated by the production of a brown coloration round the colonies, no special selective methods were applied. Subsequent work (Henderson, 1960) showed that the coloration with tannic acid was not linked with an ability to utilize compounds related to lignin and to decompose them; a similar conclusion was reached recently by Ross (1960). In the present investigations a soil enrichment technique with vanillin or *p*-hydroxybenzaldehyde as substrate was used with the aim of isolating lignin-decomposing fungi, and a survey of vegetational types similar to those studied previously carried out. This paper describes the isolation and growth of a number of yeast-like fungi which can utilize compounds related to lignin and which were not encountered in the previous studies.

METHODS

Soil samples were obtained from a heath, Scots Pine forest (actively decomposing litter and humus layers), peat moss, garden and field. With the exception of the Scots Pine litter and humus samples, which were collected by means of sterilized forceps from the appropriate exposed layer, the samples were obtained by scraping a freshly exposed face, from a depth of $6\frac{1}{2}$ in. to $\frac{1}{2}$ in. below the surface, with a sterilized scoop. The samples, three from each area, were immediately transferred to sterile jars and were thoroughly mixed before the removal of samples for the isolation of micro-organisms.

Isolation of fungi. Portions (2.5 g.) of the 'soil' samples were added to 50 ml. sterile water contained in a Waring micro-blender, which was run at top speed for

1 min. Samples (0.5 ml.) of the resulting suspensions were added to 20 ml. lots of Turfitt's medium (Turfitt, 1944) omitting cholesterol and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and adding 0.01 % (w/v) vanillin or *p*-hydroxybenzaldehyde as carbon source. After incubation at 27° for 7 days the flasks were shaken by hand for 1 min. and 1 ml. from each culture flask was transferred to a replicate flask which was incubated for a further 7 days. After incubation the flasks were again shaken by hand for one minute and 1 ml. portions of the resulting suspensions were used for the preparation of dilutions at 1/1000, 1/10,000 and 1/100,000. One ml. from each dilution was added to each of two plates of Waksman's agar (Waksman, 1922) modified by the replacement of peptone by 0.25 % (w/v) $(\text{NH}_4)_2\text{SO}_4$. The plates were incubated for 14 days at 27° and representative organisms were isolated from them during this period. The isolates were maintained in culture on potato glucose agar.

Growth experiments. Suspensions for inoculation were obtained by growing the organisms on 50 ml. modified Czapek mineral salts (NaNO_3 , 1.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g.; KH_2PO_4 , 0.5 g.; KCl, 0.25 g.; glucose, 5 g.; Bacto yeast extract (Difco Laboratories, Detroit, Michigan), 2.5 g.; water, 500 ml.) and incubating at 27° for 2 days. The resulting growth was centrifuged off, washed three times with sterile water and suspended in 10 ml. water. One ml. portions of these suspensions were added to 99 ml. water and single drops, from a 1 ml. pipette, of the resulting suspensions served as inocula.

The basal mineral salts medium for the growth experiments was the same as that used above for the preparation of inocula, but with the glucose and yeast extract omitted. It was sterilized by autoclaving at 120° for 20 min. The required substrates, sterilized by filtration, were added to give final concentrations of 0.01 % (w/v). Ten ml. lots of media were dispensed in 1 oz. vials closed with cotton-wool plugs. After inoculation the bottles were incubated on a shaking machine for 5 days at 27°.

Estimation of growth. In agitated liquid media the fungi grew in a predominantly unicellular form and growth was estimated by counting the number of viable organisms by the technique of Miles & Misra (1938). Occasionally small pellets of mycelium were formed, which led to an abnormally low count. In order to obtain zero time counts, bottles containing 10 ml. water were inoculated in a similar way to the media. One ml. samples were removed aseptically immediately from such bottles and after 1, 2, 3, 4, and 5 days of incubation from the experimental bottles. These samples were used for the preparation of dilutions in the range 1/10 to 1/10⁵, according to the amount of growth. The medium used for making the counts was potato glucose agar which, after pouring, was allowed to solidify and the plates, with the lids slightly raised, were then placed in an incubator at 60° for 2 hr. to dry the surface of the agar. Three drops from each dilution were placed on plates which were then incubated at 27° for 2 days (*Pullularia pullulans*, *Margarinomyces mutabilis*) or 3 days (*M. heteromorpha*) before counting. The volume of the drops added to the plates being known, it was possible to calculate the number of organisms/ml. in the inocula and cultures.

RESULTS

Isolation of fungi by the enrichment technique

The yield of organisms from the different areas varied markedly, there being in some cases a definite enrichment of fungi, while in others there was none. When enrichment did occur it was noticeable that most of the isolates obtained were similar morphologically. They were either mycelial yeasts or Fungi Imperfecti closely resembling these yeasts. These isolates were sent to the Centraalbureau voor Schimmelcultures, Baarn, Netherlands, for identification. A marked growth in the enrichment cultures was observed on incubating the Scots Pine litter sample in the presence of vanillin. Ten representative isolates were obtained in pure culture and were subsequently identified as *Pullularia pullulans* (6); *Trichosporon cutaneum* var. *multisporum* (1); *Phialophora aurantiaca* (1).

Two isolates (no. 8, 10) were not identical with any known species. Although not identical in all respects they were considered by the Baarn workers to belong to one species. The description of these two isolates was given as follows (personal communication):

'Elements, occurring in a young malt extract culture are very different in appearance: small cells round, about $1.2-3\ \mu$ in diameter, oval to long-oval cells $(2.8-4.5) \times (5-10)\ \mu$. True mycelium, thinly septate, is produced. After 1 week a sediment is developed and a thin pellicle. The streak culture on malt agar after 1 week at room temperature is creamy and tough, hairy all over the surface in no. 8, in no. 10 hairy in the lower part, wrinkled in the upper part of the streak. In slide cultures on potato-agar outside the coverslip: good growth of true mycelium, the cross-walls of which are thin. Small round spores develop all along the mycelium, often on denticles. Under the coverslip a pseudomycelium develops as well, with oval or elongate blastospores. Asci and ascospores have not been found. These two strains do not ferment sugars. Aerobically glucose, galactose, saccharose, maltose and lactose are assimilated, the latter latently, in the auxanographic test. Ethanol is used as an only source of carbon in the liquid medium according to Wickerham; KNO_3 is not utilized as an only source of nitrogen; external vitamins are required for development in a synthetic medium. There is no growth at 37° . Arbutin is split after 1 week at 25° . The "starch" reaction after 3 weeks of growth in a medium of low pH in shaking cultures was negative. Comparison of the assimilation of the two strains of thirty carbon sources in Wickerham's liquid medium resulted in differences only with sorbitol and erythritol.'

The heath soil sample also gave a definite enrichment when incubated in the presence of vanillin; six representative isolates were identified as *Margarinomyces heteromorpha* (2) and *M. mutabilis* (4). There was no enrichment from Scots Pine humus, while from peat moss a few colonies of *Fusarium* sp. were obtained when the samples were incubated with vanillin. Both vanillin and *p*-hydroxybenzaldehyde were used as substrates for the garden- and field-soil enrichments. No enrichment was obtained on either substrate from the field soil, but the garden soil gave an enrichment of fungi, one of which was identified as *Geotrichum candidum* and the description of the other (isolate no. 18), is as follows:

'On malt extract after 2 days at 25° a white mouldy pellicle is formed. Microscopic examination shows elongate and cylindrical arthrospores, measuring

(3-4) \times (5-13) μ , and also true mycelium 3.5-5 μ in diameter. A young potato agar slide culture reveals true mycelium 2.5-4.5 μ , and young "buds", arising on small projections, situated laterally on the mycelium, sometimes also on the joints of the arthrospores. Sometimes these "buds" are formed inside an empty cell of the mycelium. They contain an oil drop. There is no sign of development of ascospores. The organism is not capable of alcoholic sugar fermentation. Glucose and galactose are utilized as sources of carbon; sucrose, maltose, lactose and raffinose are not assimilated, neither is ethanol. KNO_3 is not used as a sole source of nitrogen; arbutin is not split. The organism needs external vitamins for growth in a synthetic medium. It will not develop on malt agar at 37°.

Growth experiments

The following isolates were used for growth experiments: *Pullularia pullulans* from Scots Pine litter; *Margarinomyces heteromorpha* and *M. mutabilis*, both from heath soil. Glucose was included as a substrate in all growth experiments, for

Table 1. *Growth of Pullularia pullulans, Margarinomyces heteromorpha and M. mutabilis on aromatic substrates*

Aromatic compounds or glucose were added to the basal growth medium (NaNO_3 , 1.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g.; KH_2PO_4 , 0.5 g.; KCl , 0.25 g.; water, 500 ml.) to give final concentrations of 0.01 % (w/v) experiments 1 to 4 or 0.01 M experiments 5 to 6. Ten ml. lots of media were used. One drop of suspension used as inocula gave zero hour counts (Miles & Misra, 1938; no. viable particles $\times 10^{-4}/\text{ml.}$): *P. pullulans*, 1.4-2.9; *M. heteromorpha*, 3.5-3.9; *M. mutabilis*, 14.3-17.3). Growth was for 5 days at 27° on a shaking machine.

Expt. no.	Substrate	Number of viable particles ($\times 10^{-5}$)/ml.		
		<i>P. pullulans</i>	<i>M. heteromorpha</i>	<i>M. mutabilis</i>
1	Control	0.82	57.0	3.1
	Glucose	13.0	390.0	95.0
	<i>p</i> -Hydroxybenzaldehyde	6.4	230.0	53.0
2	Control	1.6	28.0	6.3
	Glucose	—	—	77.0
	Ferulic acid	25.0	120.0	77.0
3	Control	2.4	28.0	9.1
	Glucose	14.0	—	41.0*
	Syringaldehyde	10.0	99.0	29.0*
4	Control	2.8	41.0	7.6
	Glucose	27.0	170.0	160.0
	Vanillin	11.0	81.0	69.0
5	Control	3.7	40.0	9.6
	Glucose	28.0	110.0	57.0*
	Benzoic acid	0	0	0
	Syringic acid	0.33*	70.0	0
	Vanillic acid	29.0	120.0	18.0*
6	Control	1.8	31.0	8.8
	Glucose	610.0	2200.0	1400.0
	<i>o</i> -Hydroxybenzoic acid	0	0	0
	<i>m</i> -Hydroxybenzoic acid	0	390.0	0
	<i>p</i> -Hydroxybenzoic acid	0	94.0	0

* Many pellets present, decreasing count.

comparative purposes. In the first four growth experiments the aromatic substrates were *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde and vanillin. Growth on glucose started immediately, while on the aromatic substrates there was generally little growth after 1 day, the rate of growth increased between 1 and 2 days and reached a maximum after 2 days. It can be seen from Table 1 that the total growth on these aromatic substrates was always considerably greater than that of the controls without added substrate. In the fifth and sixth experiments the aromatic substrates (at 0.01 M) were benzoic, syringic and vanillic acids and *o*-, *m*- and *p*-hydroxybenzoic acids. Estimations of the number of viable particles were made only on the 5th day. The results are given in Table 1. With the exception of benzoic

Table 2. *Growth of Pullularia pullulans, Margarinomyces heteromorpha and M. mutabilis on different concentrations of p-hydroxybenzaldehyde, p-hydroxybenzoic acid, vanillin and vanillic acid*

Experimental details as in Table 1, but substrates added at concentrations shown and incubation was for 6 days.

Substrate	Concentration of substrate (M)			
	0.01	0.0025	0.0014	0.001
<i>P. pullulans</i>				
<i>p</i> -Hydroxybenzaldehyde	—	—	(+)	(+)
<i>p</i> -Hydroxybenzoic acid	(+)	(+)	(+)	(+)
Vanillin	—	—	+	+
Vanillic acid	++++	++(+)	++	+
Control	—	.	.	.
<i>M. mutabilis</i>				
<i>p</i> -Hydroxybenzaldehyde	—	+	++	++
<i>p</i> -Hydroxybenzoic acid	+	++	+++	++
Vanillin	—	(+)	++(+)	++(+)
Vanillic acid	—	++	+++	+++
Control	(+)	.	.	.
<i>M. heteromorpha</i>				
<i>p</i> -Hydroxybenzaldehyde	—	+	+(+)	+(+)
<i>p</i> -Hydroxybenzoic acid	+++	++	++	++
Vanillin	(+)	(+)	++	++
Vanillic acid	+++	++	++	++
Control	(+)	.	.	.

— = no growth; (+) = small amount of growth; +, ++, +++ = relatively greater amounts of growth.

and *o*-hydroxybenzoic acids all of these substrates supported growth of *M. heteromorpha*, while *P. pullulans* grew only on syringic and vanillic acids and *M. mutabilis* grew only on vanillic acid. A further series of growth experiments used *p*-hydroxybenzaldehyde and vanillin and their corresponding acids at 0.01 M, 0.025 M, 0.0014 M and 0.001 M, the results are given in Table 2. Counts were not taken, but a visual comparison of growth was made. *P. pullulans* was least, and *M. heteromorpha* most, tolerant of the higher concentrations. *P. pullulans* gave markedly greater growth on vanillic acid than on *p*-hydroxybenzoic acid, which did not occur with the other species. There was a tendency for the acids to support growth at a higher concentration than did their aldehydes.

DISCUSSION

The organisms isolated by the soil-enrichment technique used in the present work were all of the same basic morphological types, although some are classified with the yeasts and some with the Hyphomycetes. *Trichosporon cutaneum* var. *multisporum* is a yeast, while *Phialophora aurantiaca*, *Margarinomyces heteromorpha*, *M. mutabilis* and *Geotrichum candidum* are classified in the Hyphomycetes. *Pullularia pullulans* has been assigned to both groups.

The three isolates which were examined for their ability to grow on the lignin-related aromatic compounds *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde and vanillin, were able to utilize them as sole sources of carbon. These compounds were not as readily available as glucose and there was usually a lag of about 2 days before the growth rates reached their maxima, which in each case were similar to the growth rates on glucose. Previous work (Henderson & Farmer, 1955) revealed the widespread ability of soil microfungi to grow on the above aromatic compounds, while di Menna (1959) found that a number of yeasts could also utilize them. The present studies therefore add to the list of organisms known to attack lignin-related aromatic compounds in soil and emphasize the possible role of micro-organisms in the decomposition of lignin under natural conditions.

The author is indebted to the staff of the Centraalbureau voor Schimmelcultures, Baarn, Netherlands, who identified the isolates. She wishes to acknowledge, also, the technical assistance given by Miss Dorothy Brebner and helpful advice from Dr D. M. Webley.

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The Metabolism of Aromatic Compounds Related to Lignin by some Hyphomycetes and Yeast-like Fungi of Soil

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SUMMARY

The metabolism of various lignin-related aromatic compounds by several soil Hyphomycetes and yeast-like fungi was investigated. Adaptation studies with whole organisms and cell-free extracts confirmed previously proposed metabolic pathways (Henderson & Farmer, 1955; Henderson, 1960). It was shown that protocatechuic acid is an intermediate in the metabolism of vanillin and ferulic acid. Protocatechuic acid oxidase activity of cell-free extracts of *Pullularia pullulans* was found to be stimulated by ferrous ions and to depend on -SH groups.

INTRODUCTION

The studies reported in the previous paper (Henderson, 1961) were extended by investigation of the metabolism of several lignin-related aromatic compounds by yeast-like fungi of soil. Techniques previously employed with other soil microfungi for studying oxygen uptakes (Henderson, 1956) and for the isolation of intermediate products of metabolism (Henderson, 1957) were used. Cell-free extracts of *Pullularia pullulans* were found to possess protocatechuic acid oxidase activity, a property which proved to be very useful for indicating the adaptation to protocatechuic acid of organisms which had been exposed to several aromatic compounds. Thus these compounds could be associated in metabolic pathways which finally passed through protocatechuic acid.

METHODS

Organisms. These were isolated by a soil-enrichment technique (Henderson, 1961). They were *Pullularia pullulans*, *Margarinomyces heteromorpha*, *Margarinomyces mutabilis* (2 isolates), *Phialophora aurantiaca*, *Geotrichum candidum*, *Fusarium* sp. and unidentified isolates no. 8 and 10.

Production of organisms. The fungi were grown on the basal mineral salts medium previously used (Henderson, 1961), to which were added glucose, Difco yeast extract and aromatic compounds as required. For metabolic experiments in the Warburg apparatus and for the large-scale metabolic experiments the growth medium contained 1% (w/v) glucose and 0.5% (w/v) yeast extract. The medium was dispensed in 40 ml. lots in 250 ml. conical flasks; incubation was for 3 days at 27° on a shaking machine (Webley & Duff, 1955). The growth was then harvested and washed three times with distilled water. When the organisms were to be adapted to different substrates the harvesting and washing were carried out aseptically, and the washed

organisms transferred to 250 ml. conical flasks containing 50 ml. 0.067 M-phosphate buffer (Clark, 1928) at pH 5.2, and substrate at 0.001 M. Control organisms were transferred to buffer only. After incubation overnight on the shaking machine the organisms were again harvested and washed.

In the production and adaptation of cells of *Pullularia pullulans* for preparation of crude cell-free extracts organisms were obtained from 160 ml. lots of medium containing 2% (w/v) glucose \pm 0.001 M-aromatic compounds. The media were dispensed in 1 l. culture flasks (Jobling Cat. no. 1410). Since the fungus would not grow in the presence of catechol or *o*-hydroxybenzoic acid, when these substances were being investigated it was grown on the basal mineral salts medium + 2% (w/v) glucose, and the organisms were adapted by transferring them aseptically to phosphate buffer (pH 5.2) + 0.001 M solutions of these compounds and then incubating overnight. Control organisms were transferred to buffer only.

Manometry. Initially the respiration of *Pullularia pullulans* and *Margarinomyces mutabilis* in the presence of vanillin was tested over a range from pH 4 to pH 8 (McIlvaine's buffer). Since neither species was sensitive over this pH range it was decided to use 0.067 M-phosphate buffer (pH 5.2) as in previous experiments with soil fungi (Henderson, 1956). In experiments with whole organisms 1.0 ml. of suspension (containing about 7 mg. dry wt. organism/ml. and 0.5 ml. buffer were added to the main compartment of each Warburg flask, 0.2 ml. 5% (w/v) KOH to the centre cup and 0.5 ml. of 0.01 M solutions of substrates (except ferulic acid which was 0.003 M) to the side-arm. Acid substrates were neutralized with sodium hydroxide. Experiments were conducted at 30° in air. Readings were taken at 30 min. intervals for 3½ hr., substrates being tipped in from the side-arms after 30 min. In experiments with cell-free extracts 0.5 ml. of the extracts and 1.0 ml. of 0.067 M-phosphate buffer (pH 7) were added to the main compartment and 0.02 M solutions of substrates were used. Substrates were tipped from the side-arms at 0 hr. and experiments were run for 4 hr. before carrying out β -ketoadipic acid estimations. Otherwise conditions were the same as those for experiments with whole organisms.

Large-scale metabolic experiments. Suspensions containing about 11 mg. dry wt. *Pullularia pullulans*/ml., 10 mg. dry wt. *Margarinomyces heteromorpha*/ml. and 6 mg. dry wt. *M. mutabilis*/ml. were used. Three ml. of suspension were added to 25 ml. 0.01 M substrate solution in 100 ml. conical flasks (except in the case of ferulic acid when 25 ml. 0.003 M substrate solution was used). The aldehydes were sterilized by filtration through sintered glass and the acids by autoclaving at 120° for 20 min. at pH 6.5. After addition of the suspensions the flasks were incubated on the shaking machine and one flask of each substrate was removed after 5 and 22 hr. The growth was removed by centrifugation, the supernatant fluid acidified and extracted 3 times with 10 ml. ether. The ether was removed by evaporation and the extracts were dissolved in a few drops of absolute ethanol. Samples were applied to Whatman no. 1 filter papers which were developed with *n*-butanol + ammonia (sp.gr. 0.880) + water (80 + 5 + 15 vol.) for 16 hr. at 21° by the descending method. They were sprayed with diazotized sulphanilic acid (Bray, Thorpe & White, 1950).

Cell-free extracts. Crude extracts of organisms were prepared according to the method of McIlwain (1948). The organisms were harvested, washed three times and ground in a pestle and mortar with three times their wet weight of alumina (H fine Aloxite, The Carborundum Company Ltd., Manchester, 17). For this purpose

approximately 2 g. wet wt. organism were used. The crushed organisms were extracted with 3 ml. of 0.067 M-phosphate buffer (pH 7). The crude extract was freed from alumina by centrifugation at 1600 g for 10 min. After the crude extract had been centrifuged at 20,000 g for 30 min. precipitation with $(\text{NH}_4)_2\text{SO}_4$ was sometimes carried out according to Dagley & Patel (1957). The precipitates obtained were separated by centrifugation at 20,000 g for 30 min. The supernatant fluids were removed and the precipitates dissolved in a volume of 0.02 M-phosphate buffer (pH 7) equal to the volumes of crude extract from which they were prepared.

β -Keto adipic acid estimation. The presence of β -keto adipic acid was detected by the Rothera test (Rothera, 1908). The amount of acid present was estimated by decarboxylation with 4-aminoantipyrine and measuring the carbon dioxide evolved (Sistrom & Stanier, 1953).

RESULTS

Oxygen uptake by fungi in the presence of aromatic substrates

In experiment 1 (Table 1) oxygen uptakes in the presence of *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde and vanillin were measured. All species metabolized these substrates to some extent, but there was considerable variation

Table 1. *Oxygen uptake by fungi in presence of aromatic compounds*

Added to each flask were: 1.0 ml. cell suspension (equiv. about 7 mg./ml. dry wt.) + 0.5 ml. phosphate buffer (pH 5.2) in the main compartment; 0.2 ml. 5% (w/v) KOH in centre cup; 0.5 ml. 0.01 M solutions of substrates (except ferulic acid which was 3×0.001 M) or 0.5 ml. water in side-arm. In Expt. 2 the cells were starved by shaking overnight in phosphate buffer (pH 5.2). The figures refer to oxygen uptake over the 3 hr. period following the addition of substrates.

Expt. 1.	Substrate				
	<i>p</i> -Hydroxy- benzaldehyde	Ferulic acid	Syringaldehyde	Vanillin	Control
	(oxygen uptake, $\mu\text{l.}$)				
Fungus					
<i>Fusarium</i> sp.	400	589	314	251	213
<i>G. candidum</i>	174	94	131	142	88
<i>M. heteromorpha</i>	349	344	432	526	199
<i>M. mutabilis</i> (a)	489	328	558	635	240
<i>M. mutabilis</i> (b)	444	263	403	508	191
<i>P. aurantiaca</i>	265	307	455	553	267
<i>P. pullulans</i>	198	330	157	287	93
Isolate no. 8	651	298	541	564	201
Isolate no. 10	142	101	92	92	85

Expt. 2.	Substrate				
	<i>p</i> -Hydroxy- benzoate	Syringate	Vanillate	Proto- catechuate	Control
	(oxygen uptake, $\mu\text{l.}$)				
Fungus					
<i>Fusarium</i> sp.	151	53	129	137	40
<i>G. candidum</i>	81	110	91	79	87
<i>M. heteromorpha</i>	373	149	290	276	39
<i>M. mutabilis</i> (a)	232	206	241	250	88
<i>M. mutabilis</i> (b)	208	187	213	213	79
<i>P. aurantiaca</i>	155	195	197	302	142
<i>P. pullulans</i>	144	129	352	324	62
Isolate no. 8	118	67	64	132	63
Isolate no. 10	161	90	82	142	67

in the oxygen uptakes by different isolates on different substrates. These substrates were metabolized without any lag period. In Expt. 2 (Table 1) oxygen uptakes in the presence of sodium *p*-hydroxybenzoate, syringate, vanillate and protocatechuic acid were recorded. Since the rates of oxygen uptake with this group of substrates were considerably slower than with the first group, overnight starvation of the organisms in buffer was necessary to decrease the endogenous respiration. This made the differences in oxygen uptakes between the control and experimental flasks more distinct. In contrast to Expt. 1 there were lag periods for all these substrates before the oxygen uptakes reached their maximum rate, as found previously for other soil fungi (Henderson, 1956). The lag period with *Pullularia pullulans* on protocatechuic acid was eliminated by previous incubation with vanillin, vanillic acid, *p*-hydroxybenzaldehyde or *p*-hydroxybenzoic acid (Fig. 1, *a*, *b*). Incubation with syringic acid did not appear to lead to adaptation to protocatechuic acid. Previous incubation with ferulic acid eliminated the lag period with vanillic acid.

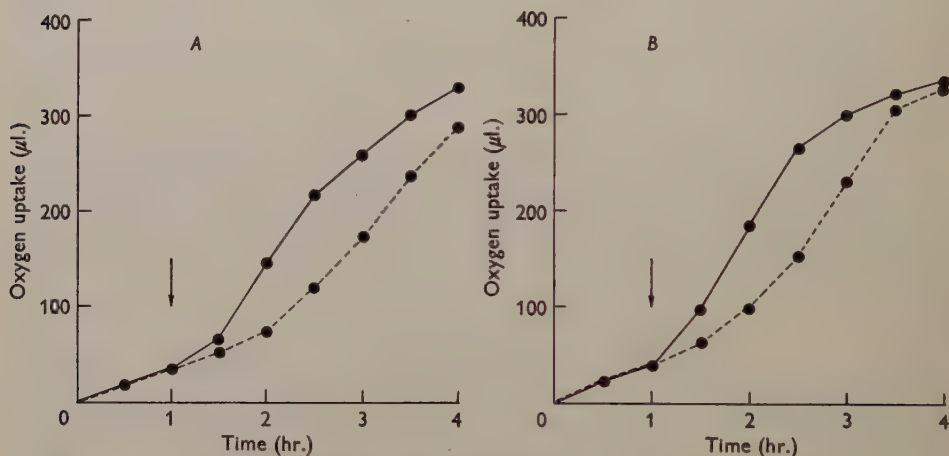


Fig. 1, *A*, *B*. Oxygen uptake by *Pullularia pullulans* in the presence of protocatechuic acid. Added to each flask were: 1.0 ml. organism suspension (equiv. about 7 mg. dry wt./ml.) + 0.5 ml. phosphate buffer (pH 5.2) in main compartment; 0.2 ml. 5% (w/v) KOH in centre cup; 0.5 ml. 0.01 M solution of protocatechuic acid in side arm. — = adapted cells; --- = non-adapted cells. *A*: cells adapted to vanillin; *B*: cells adapted to *p*-hydroxybenzaldehyde. Protocatechuic acid added where indicated by arrow.

Intermediate products of metabolism detected in large-scale metabolic experiments

All of the aromatic compounds tested were attacked by the fungi and some were so rapidly metabolized that no trace of them could be found when incubation proceeded beyond 2 days. The principal intermediate products detected (see Table 2) included those found previously (Henderson & Farmer, 1955; Henderson, 1957): *p*-hydroxybenzoic acid from *p*-hydroxybenzaldehyde, vanillic acid from vanillin and ferulic acid, syringic acid from syringaldehyde, the hydroxybenzoic acids from the corresponding mono-methoxybenzoic acids, protocatechuic acid from *p*-hydroxybenzoic acid, *p*-hydroxybenzoic and protocatechuic acids from benzoic acid and vanillic acid from 3:4-dimethoxybenzoic acid. No intermediates

Table 2. *Principal intermediate products of metabolism of aromatic compounds detected by paper chromatography*

Suspensions of organisms were added to 25 ml. of 0.01 M solutions of the substrates and incubated for 5 or 22 hr. The cell material was removed and the supernatant fluids extracted with ether. The extracts were examined by paper chromatography. Papers were developed with *n*-butanol+ ammonia (sp.gr. 0.880)+ water (80+5+15) for 16 hr. at 21°, by the descending method. They were sprayed with diazotized sulphanilic acid. R_F values were calculated from leading edge of spots.

Substrate	Organism					
	<i>P. pullulans</i> , product or colour	R_F	<i>M. heteromorpha</i> , product or colour	R_F	<i>M. mutabilis</i> , product or colour	R_F
Benzoic acid	<i>p</i> -Hydroxybenzoic acid	0.267	<i>p</i> -Hydroxybenzoic acid	0.262	<i>p</i> -Hydroxybenzoic acid	0.283
	Protocatechuic acid	0.057			Protocatechuic acid	0.058
Ferulic acid	Vanillic acid	0.133	Vanillic acid	0.125	Vanillic acid	0.117
<i>p</i> -Hydroxybenzaldehyde	<i>p</i> -Hydroxybenzoic acid	0.141	<i>p</i> -Hydroxybenzoic acid	0.131	<i>p</i> -Hydroxybenzoic acid	0.175
	? Yellow	0.885			? Yellow	0.836
	? Yellow	0.784				
<i>o</i> -Hydroxybenzoic acid	? Yellow	0.183	? Yellow	0.152	Gentisic acid	0.239
<i>m</i> -Hydroxybenzoic acid	—		Protocatechuic acid	0.042	? Yellow	0.162
<i>p</i> -Hydroxybenzoic acid	Protocatechuic acid	0.046	Protocatechuic acid	0.062	Protocatechuic acid	0.025
<i>o</i> -Methoxybenzoic acid	<i>o</i> -Hydroxybenzoic acid	0.584	<i>o</i> -Hydroxybenzoic acid	0.474	<i>o</i> -Hydroxybenzoic acid	0.492
<i>m</i> -Methoxybenzoic acid	<i>m</i> -Hydroxybenzoic acid	0.223	—		<i>m</i> -Hydroxybenzoic acid	0.283
<i>p</i> -Methoxybenzoic acid	<i>p</i> -Hydroxybenzoic acid	0.179	<i>p</i> -Hydroxybenzoic acid	0.140	<i>p</i> -Hydroxybenzoic acid	0.173
3:4-Dimethoxybenzoic acid	Vanillic acid	0.122	—		Vanillic acid	0.126
2:4-Dimethoxybenzoic acid	? Orange-Yellow	0.107	? Yellow	0.272	? Orange	0.320
			? Orange	0.129	? Pink-Orange	0.113
Syringaldehyde	Syringic acid	0.104				
	? Red	0.827				
Vanillin	Vanillic acid	0.129	Vanillic acid	0.143	Vanillic acid	0.179
	Vanillyl alcohol	0.797			? Orange	0.774

Colours. Hydroxybenzoic acids: yellow; protocatechuic acid: pink-white; vanillyl groups: orange; gentisic acid: grey-white; syringic acid: red. — = No spots obtained.

from 2:4-dimethoxybenzoic acid were identified. Gentisic acid was formed by *Margarinomyces mutabilis* from *o*-hydroxybenzoic acid. It was identified as follows. A solution of *o*-hydroxybenzoic acid was incubated in the presence of *M. mutabilis* for 2 days, the organisms removed and the supernatant fluids acidified and extracted with ether. The extract, after application to paper chromatograms, was developed with *n*-propanol+ ammonia (sp.gr. 0.880)+ water (80+5+15 vol.) and sprayed with diazotized sulphanilic acid. Pure gentisic acid was run as a standard along with the extract. Strips containing standard spots were cut from each side of the paper and the position of gentisic acid in them was located by spraying with diazotized sulphanilic acid. Strips from the centre of the paper, one containing the

extract and the other gentisic acid, were cut out and the areas in them, corresponding to the location of gentisic acid in the test strips, were removed and eluted with 2.5 ml. 0.067 M-phosphate buffer (pH 7). The solutions obtained were examined by ultraviolet spectrometry and were found to have corresponding maxima at 328 m μ in acid solution, 317 and 259 m μ in alkaline solution and 320 m μ in neutral solution, confirming that gentisic acid was present in the extract from the experimental solution.

A number of spots of high R_F values which had not been obtained previously were noted. These included vanillyl alcohol which was identified in an extract obtained after incubating a solution of vanillin in the presence of a suspension of *Pullularia pullulans* for 2 days. After removal of the organisms the supernatant fluid was extracted with ether without previous acidification. The extract yielded a large orange spot of R_F 0.812 and a faint orange spot of R_F 0.085 when developed with butanol + ammonia + water (80 + 5 + 15) and sprayed with diazotized sulphathiazole. The former spot corresponded to an unidentified spot obtained in extracts after incubation for 5 and 22 hr.; the latter spot was vanillic acid. Since the extract contained no residual vanillin it could be used directly for analysis by infrared spectrometry; its spectrum was found to be identical with that of vanillyl alcohol. A spot corresponding to vanillyl alcohol was also obtained on developing the extract from *Margarinomyces mutabilis* cultures on vanillin. Spots of high R_F value from extracts of cultures of the three species on *p*-hydroxybenzaldehyde and of *P. pullulans* on syringaldehyde may also have been the corresponding alcohols, but standards were not available for comparison.

These results indicate that the metabolism was basically similar to that of the filamentous fungi investigated previously, but there was evidence of a strong reducing mechanism in these organisms which led to the formation of alcohols from aldehydes. Vanillyl alcohol was produced even when a stream of air was bubbled through a flask containing *Pullularia pullulans* and vanillin, but no spot of high R_F value was obtained when *p*-hydroxybenzaldehyde was similarly treated.

Protocatechuic acid oxidase activity in cell-free extracts

Preliminary Warburg experiments showed that cell-free extracts from adapted organisms took up oxygen in the presence of protocatechuic acid and that β -keto-adipic acid was produced, as indicated by the Rothera test (Rothera, 1908). Extracts from non-adapted organisms showed negligible protocatechuic acid oxidase activity. In subsequent experiments protocatechuic acid oxidase activity was determined by estimating the β -keto-adipic acid which accumulated after incubation of extracts with protocatechuic acid.

Table 3 shows that protocatechuic acid oxidase activity was well developed in extracts of those organisms which had been exposed to *m*- and *p*-hydroxybenzoic and vanillic acids, as well as to protocatechuic acid. These results indicate that protocatechuic acid was an intermediate product in the metabolism of these acids. Activity was very low in organisms which had been exposed to syringic acid, signifying that protocatechuic acid was not involved in its metabolism. It can also be seen that exposure to *o*-hydroxybenzoic acid and to catechol did not lead to the adaptation of organisms to protocatechuic acid. However, exposure to these substrates led to adaptation to catechol and *cis-cis*-muconic acid, while organisms which had been exposed to protocatechuic acid were negative in this respect.

Table 3. *β -Keto adipic acid production by crude extracts of Pullularia pullulans from protocatechuic acid, cis-cis-muconic acid and catechol*

Before extraction half of the organisms were adapted to various substrates. Added to each flask were: 1.0 ml. phosphate buffer (pH 7)+ 0.5 ml. cell extract in the main compartment; 0.2 ml. 5% (w/v) KOH in the centre cup; 0.5 ml. 0.02 M solutions of substrates (10 μ mole) in the side-arm. Experiments were run for 4 hr. at 30°. β -Keto adipic acid was estimated by decarboxylation with aminoantipyrine.

Adapting substrate	Substrate					
	Protocatechuic acid		<i>cis-cis</i> -Muconic acid		Catechol	
	Extract: from adapted (A) or non-adapted (NA) organism					
	A	NA	A	NA	A	NA
	β -keto adipic acid produced (μ mole)					
Catechol	1.4	1.4	6.5	1.8	8.8	1.4
<i>o</i> -Hydroxybenzoic acid	1.2	0.9	5.0	0.9	8.8	1.1
<i>m</i> -Hydroxybenzoic acid	6.3	1.5	—	—	—	—
<i>p</i> -Hydroxybenzoic acid	5.9	0.7	—	—	—	—
Protocatechuic acid	8.2	0.7	0.8	0.7	0.4	0.5
Syringic acid	1.9	1.3	—	—	—	—
Vanillic acid	7.5	1.8	—	—	—	—

Protocatechuic acid oxidase activity in crude cell-free extracts was partly inhibited by 1.4×10^{-4} M-*p*-chloromercuribenzoate (CMB), but activity was retained when glutathione at 1.3×10^{-3} M was present. When the concentration of CMB was increased to 5.6×10^{-4} M the enzyme activity was completely inhibited, but was restored by 1.3×10^{-3} M glutathione (see Table 4). When crude cell-free extracts were precipitated with $(\text{NH}_4)_2\text{SO}_4$ and the precipitates dissolved in phosphate buffer according to Dagley & Patel (1957), the resulting solutions showed considerable protocatechuic acid oxidase activity, which was inhibited by 5.6×10^{-4} M-CMB and

Table 4. *Influence of p-chloromercuribenzoate and glutathione on protocatechuic acid oxidase activity in extracts of Pullularia pullulans*

Activity was measured by β -keto adipic acid production. Organisms were adapted to protocatechuic acid before extraction (crude extract) and precipitation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dissolved in 0.02 M-phosphate buffer (pH 7; $(\text{NH}_4)_2\text{SO}_4$ precipitate). Added to each flask were: 1.0 ml. phosphate buffer (pH 7)+ 0.5 ml. cell extract+ 0.5 ml. *p*-chloromercuribenzoate (CMB), to give a final concentration of 1.4×10^{-4} M or 5.6×10^{-4} M, or 0.5 ml. water + 0.5 ml. glutathione, to give a final concentration of 1.3×10^{-3} M in the main compartment, or 0.5 ml. water; 0.2 ml. 5% (w/v) KOH in the centre cup; 0.5 ml. 0.02 M-protocatechuic acid (10 μ mole) in the side arm. Experiments were run for 4 hr. at 30°. β -Keto adipic acid was estimated by decarboxylation with aminoantipyrine.

Addition	Crude extract		(NH ₄) ₂ SO ₄ precipitate
	Expt. 1	Expt. 2	
	<i>β</i> -keto adipic acid produced (μmole)		
None	4.5	2.3	1.2
CMB 1.4 × 10 ⁻⁴ M	2.2	—	—
CMB 5.6 × 10 ⁻⁴ M	—	0	0
CMB + Glutathione	5.4	3.4	1.4

— = Not tested

restored by 1.3×10^{-3} M-glutathione (Table 4). Dialysis against 0.022 M-phosphate buffer (pH 6.9) for 16 hr. decreased the activity of crude extracts and was partly restored by 0.001 M- FeSO_4 . The results were as follows ($\mu\text{mole } \beta\text{-ketoadipic acid}$): crude extract, 4.1; dialysed extract, 1.2; dialysed extract + FeSO_4 , 2.3; dialysed extract control, 0. No consistent results were obtained about the influence of glutathione or ferrous ion on the activity of untreated crude extracts, but the activity of solutions of ammonium sulphate precipitates was increased by the presence of 1.6×10^{-3} M-glutathione, or 0.001 M- FeSO_4 or FeCl_3 (see Table 5).

Table 5. *Influence of glutathione and various ions on protocatechuic acid oxidase activity in extracts of Pullularia pullulans after treatment with $(\text{NH}_4)_2\text{SO}_4$*

Activity was measured by $\beta\text{-ketoadipic acid}$ production. Organisms were adapted to protocatechuic acid before extraction which was followed by precipitation with $(\text{NH}_4)_2\text{SO}_4$ and solution of the precipitates in 0.02 M-phosphate buffer, (pH 7). Added to each flask were: 1.0 ml. phosphate buffer (pH 7) + 0.5 ml. cell extract + 0.5 ml. glutathione (to give a final concentration of 1.6×10^{-3} M) or 0.5 ml. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, or $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ solution (to give a final concentration of 0.001 M) in the main compartment; 0.2 ml. 5% (w/v) KOH in the centre cup; 0.5 ml. 0.02 M-protocatechuic acid (10 μmole) in the side arm. Experiments were run for 4 hr. at 30° . $\beta\text{-Ketoadipic acid}$ was estimated by decarboxylation with aminoantipyrine.

Addition	Expt. 1	Expt. 2
	$\beta\text{-ketoadipic acid produced}$ (μmole)	
None	0.3	1.5
Glutathione	2.0	—
FeSO_4	—	4.5
FeCl_3	—	3.0
MnSO_4	—	1.1
ZnSO_4	—	1.3

— = Not tested

DISCUSSION

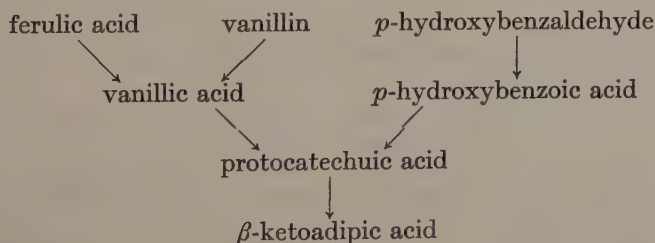
The replacement experiments showed that the soil organisms used in the present work metabolize aromatic compounds in essentially the same way as did those studied previously (Henderson & Farmer, 1955; Henderson, 1960). The principal difference lay in the reduction of vanillin to vanillyl alcohol and possibly also of *p*-hydroxybenzaldehyde and syringaldehyde to their corresponding alcohols. At the same time, oxidation of the aldehydes with the formation of the corresponding acids was proceeding. This may be compared with the anaerobic dismutation of aldehydes by *Acetobacter* spp. investigated by Molinari (1929). He found that benzaldehyde, cinnamaldehyde and anisaldehyde were dismuted to their respective acids and alcohols. In contrast Bachman, Dagoon & John (1960) obtained quantitative conversion of *o*-hydroxybenzoic to the corresponding alcohol by *Neurospora crassa*, while *Polystictus versicolor* reduced certain aromatic acids to the corresponding aldehydes and alcohols (Farmer, Henderson & Russell, 1959). The acids and alcohols found in the present work were intermediate products of metabolism and with time all trace of phenolic compounds disappeared. A further difference from previous findings was the metabolism of *o*-hydroxybenzoic acid through gentisic acid by *Margarinomyces mutabilis*. Catechol was previously detected as an intermediate

product of the metabolism of *o*-hydroxybenzoic acid by fungi (Henderson, 1960) and is the most common intermediate in bacterial metabolism. However, Mitoma, Posner, Reitz & Udenfriend (1956) obtained gentisic acid from *o*-hydroxybenzoic acid when it was subjected to a hydrolysing system found in liver microsomes.

The results from the present experiments showed that protocatechuic acid was an intermediate product in the metabolism of *p*-hydroxybenzoic, *m*-hydroxybenzoic and benzoic acids, while *p*-hydroxybenzoic acid is itself an intermediate in the metabolism of *p*-hydroxybenzaldehyde and *p*-methoxybenzoic acid. Previously the formation of protocatechuic acid, by other fungi, from *m*-hydroxybenzoic acid (Henderson, 1960) and from *p*-hydroxybenzoic acid (Henderson, 1957) was demonstrated; protocatechuic acid is also well known as an intermediate in the metabolism of various aromatic compounds by bacteria. Thus the metabolism of protocatechuic acid itself appears to be a focal point in the metabolism of aromatic compounds. The principal aim of the present respiration studies with whole organisms and with cell-free extracts was, therefore, directed towards linking the metabolism of various compounds with protocatechuic acid and studying the metabolism of this acid itself.

The morphology of the organisms used here made them very suitable for respiration experiments. In addition, *Pullularia pullulans*, which was selected for further work with cell-free extracts, lent itself very well to this type of work. Adaptation studies with whole organisms confirmed the postulated pathways of metabolism leading eventually to protocatechuic and β -ketoadipic acids. Previous incubation with vanillin, vanillic acid, ferulic acid, *p*-hydroxybenzaldehyde or *p*-hydroxybenzoic acid resulted in the elimination of the lag period which preceded the oxidation of protocatechuic acid, thus linking the metabolism of the compounds in a common pathway. Although it was not demonstrated by isolation from cultures on vanillin or vanillic acid that protocatechuic acid was an intermediate in their metabolism, in previous experiments with *Aspergillus niger* (Henderson, 1960) an indication of its production from vanillic acid was obtained. Also, vanillic acid is known to induce the synthesis of protocatechuic acid oxidase in *Neurospora crassa* (Gross & Tatum, 1955). These authors found, however, that vanillic acid was not metabolized by their organism, in contrast to the fungi studied in the present work which not only oxidized it but could grow on it as sole source of carbon.

Experiments with crude cell-free extracts, in which β -ketoadipic acid formation was used to indicate protocatechuic acid oxidase activity, confirmed the above results. They can be summarized as follows:



o-Hydroxybenzoic acid is frequently metabolized through catechol (Walker & Evans, 1952; Henderson, 1960) which is further metabolized to yield *cis-cis*-muconic

acid (Stanier & Hayaishi, 1951) and β -ketoadipic acid (Evans & Smith, 1951). The results obtained with *Pullularia pullulans* indicated that it follows the *o*-hydroxybenzoic acid \rightarrow catechol \rightarrow *cis-cis*-muconic acid \rightarrow β -ketoadipic acid pathway. The course of metabolism of syringic acid was not elucidated. Protocatechuic acid oxidase activity in organisms which had been exposed to syringic acid was very low indeed, indicating that protocatechuic acid is not produced during metabolism of syringic acid. In being stimulated by FeSO_4 this protocatechuic acid oxidase resembles those of *Neurospora* sp. (Ottey & Tatum, 1956) and of *Pseudomonas* sp. (Dagley & Patel, 1957) and not that of a soil pseudomonad studied by Ribbons & Evans (1960) or that of liver (Stanier & Ingraham, 1954). The occurrence of -SH groups in the enzyme was demonstrated by its inhibition by *p*-chloromercuribenzoate, which was annulled by glutathione. Protocatechuic acid oxidase of liver (Stanier & Ingraham, 1954) was also inhibited by *p*-chloromercuribenzoate.

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Bacteriophages of *Bacillus cereus* and of Crystal-forming Insect Pathogens Related to *B. cereus*

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SUMMARY

Four phages isolated from bacteria of the *Bacillus cereus* group are described. They show cross-reactions between crystal-forming and non-crystal-forming strains which are deficient in ability to produce a lecithinase C. The implications of this finding are discussed.

INTRODUCTION

Hannay's rediscovery of the parasporal body in the insect pathogen *Bacillus thuringiensis* (Hannay, 1953) and the subsequent identification of this crystalline protein as the toxin of the organism (Angus, 1954) have stimulated considerable interest in crystal-synthesizing aerobic spore-formers and several workers have discussed the relationship between these organisms and *B. cereus* (Toumanoff, 1956; Le Corroller, 1958; Heimpel & Angus, 1958). Most of the crystal-formers produce an active lecithinase C, as do the majority of strains of *B. cereus*; but two strains, the so-called *B. entomocidus* var. *entomocidus* and *B. entomocidus* var. *subtoxicus* of Heimpel & Angus (1958) are distinguished from the rest by absence of lecithinase activity and by failure to produce acetylmethylcarbinol.

During work with *Bacillus entomocidus* var. *entomocidus* phage plaques appeared spontaneously in slope cultures and a study of the phage responsible, and subsequently of others, was undertaken in an attempt to throw some light on the inter-relationships of this group of bacteria.

METHODS

Bacteria. The work involved 21 typical lecithinase-producing strains of *Bacillus cereus*, 12 lecithinase-producing strains of *B. cereus* var. *mycoides* and 13 lecithinase-producing crystal-formers. Two lecithinase-negative crystal-formers, *B. entomocidus* var. *entomocidus* and *B. entomocidus* var. *subtoxicus* were supplied by Dr C. L. Hannay and a further strain, G2, was isolated from larvae of *Galleria mellonella*. A crystal-forming organism which produced a very small amount of lecithinase C, G1, was isolated from the same stock of larvae at an earlier date. Three lecithinase-negative strains of *B. cereus* (strains 634, 826, 827) were obtained from Dr Ellen Garvie (see Stone, 1952) and had originally been isolated from milk. A lecithinase-negative strain of *B. cereus* var. *mycoides*, strain A.C., was a laboratory stock culture of uncertain origin.

Lecithinase. Lecithinase production was detected by growth on egg-yolk agar. Most of the organisms studied were active lipase producers and this may lead to

confusion when egg-yolk agar is used to detect lecithinase activity; for this reason results were checked with the lecithin agar described by Willis (1960).

Acetylmethylcarbinol production. Ability to produce acetylmethylcarbinol was examined by the method described by Smith, Gordon & Clark (1952).

Production of acid from carbohydrates. Ability to produce acid from carbohydrates was tested in the presence of ammonia-N on the solid medium of Smith *et al.* (1952) as used by Heimpel & Angus (1958) when studying these organisms.

Media. Cultures for the production of phage stocks and for phage sensitivity testing were grown, usually from spore inocula, on 1% (w/v) nutrient agar plates containing 1% (w/v) peptone and 0.5% (w/v) NaCl. Comparison with several other growth media showed that this relatively poor medium gave the most readily visible plaques.

Isolation of phages. Four phages were isolated and used in the investigation. When crude filtrates of *Bacillus entomocidus* var. *entomocidus* were spotted on to lawns of the same organism the plaques formed were all clear and indistinguishable from one another, but on lawns of the subtoxicus variety two types of plaque were formed: (i) clear plaques from which were isolated phage A which gave similar clear plaques on the variety *entomocidus*; (ii) turbid plaques yielding phage B which also gave clear plaques on the *entomocidus* variety.

When 7-day-old broth cultures of *Bacillus entomocidus* var. *subtoxicus* are streaked on to agar plates many of the resulting colonies are phantom forms (Nungester, 1929) collapsing after incubation for 2 days to form thin flat transparent 'ghost' colonies. The organisms remain in this form indefinitely on subculture and the effect is probably due to phage although attempts to demonstrate this conclusively have so far failed. When filtrates of broth cultures of a phantom variant of this organism were spotted on to lawns of organism G 1, a few discrete phage plaques were formed from which a further phage, phage C, was isolated.

Cultures of the lecithinase-negative *Bacillus cereus* strain 826 (see Stone, 1952) often showed spontaneously developing phage plaques which yielded the fourth phage, phage D.

Phage A was routinely propagated on *Bacillus entomocidus* var. *entomocidus*, phage B on *B. entomocidus* var. *subtoxicus*, phage C on organism G 1 and phage D on *B. cereus* 826. The propagating strains were also used as indicator strains when titres of phage preparations were being determined.

Phage sensitivity tests. As mentioned by McCloy (1951) some strains of *Bacillus cereus* produce colicine-like agents whose effects can simulate confluent phage lysis at low dilutions. This is also true of many crystal-formers and the effects of these agents were readily seen when undiluted filtrates of phage preparations were dropped on to plates inoculated with test organisms. Such non-specific lysis was never seen when phage preparations were diluted 1/10 before use; sensitivity tests were therefore carried out at this dilution. Positive results observed at phage concentrations which gave confluent lysis were always confirmed by the production of separate phage plaques at higher dilutions.

Phage stocks. Phages were purified by picking from single plaques on the routine propagating strain at least four successive times and grown by spreading a few drops of a sterile-filtered preparation on agar plates the surfaces of which were inoculated with cultures of phage-sensitive organisms, and incubating overnight at

30°. The resulting growth was harvested into a few ml. of peptone water and the suspension sterilized by passing it through a Hemming centrifugal filter (Beaumaris Instrument Co. Ltd., Rosemary Lane, Beaumaris, Anglesey). Phage preparations containing 10^{10} or 10^{11} plaque-forming units/ml. on the corresponding indicator strains were readily prepared by this method which gave, on the whole, higher titres than fluid culture techniques.

Titration of phage. Rough estimates of numbers of phage particles were made by the modified Miles & Misra technique described by McCloy (1958). Accurate titrations were made by the soft agar-layer method described by Adams (1959). In both methods the plates were inoculated with spore suspensions of the indicator organism produced by harvesting 10-day-old agar slope cultures in sterile distilled water and pasteurizing the resulting suspensions at 75° for 15 min. Control plates without added phage were always set up as a check against spontaneous phage lysis. With some organisms indicator plates prepared in this way always showed spontaneous phage plaques, but phage was usually absent from 16 hr. broth cultures grown from single plaque-free colonies; such cultures were used to inoculate titration plates instead of the usual spore suspensions.

RESULTS

Acetylmethylcarbinol production

Acetylmethylcarbinol was produced by all the lecithinase-positive strains of *Bacillus cereus*, *B. cereus* var. *mycoides* and the crystal-formers. Steinhaus (1951) and Heimpel & Angus (1958) reported that the two varieties of *B. entomocidus* did not produce acetylmethylcarbinol and Dr Ellen Garvie (personal communication) has found the same for *B. cereus* 634. These negative reactions were confirmed. However, the lecithinase negative *B. cereus* var. *mycoides* strain A.C., *B. cereus* strains 826 and 827 and crystal-formers G1 and G2 were all strongly positive.

Lecithinase activities

The majority of strains of *Bacillus cereus*, *B. cereus* var. *mycoides* and of the crystal-formers were strong lecithinase producers, the zones of turbidity spreading well beyond the margins of colonies on egg-yolk and lecithin agars. *B. cereus* 634 and 826, *B. cereus* var. *mycoides* A.C., the two varieties of *B. entomocidus* and crystal-former G2 were negative in the lecithinase test. Feeble lecithinase production leading to a weak zone of turbidity below the colony and scarcely extending beyond its margin was detected with *B. cereus* 827 and with crystal-former G1.

Phage sensitivities

None of the strongly lecithinase-positive organisms (*Bacillus cereus*, *B. cereus* var. *mycoides* or crystal-formers) was susceptible to any of the four phages. The sensitivity patterns of the other strains are summarized in Table 1, which also shows the results of tests for the production of lecithinase and acetylmethylcarbinol and certain sugar fermentations, since these characters are used by Heimpel & Angus (1958) in the classification of the group. Table 2 shows the plating efficiencies of the phages when tested against the different strains as compared with a standard figure of 100 plaque-forming units for the reaction between each phage and the organism on which it was grown.

Table 1. *Phage sensitivities and other characteristics of Bacillus strains*

Organism	Sensitivity to phage				Protein crystal formation	Lecithinase production	Acetylmethyl-carbinol production	Production of acid from				
	A	B	C	D				Xylose	Arabinose	Glucose	Trehalose	Laevulose
<i>B. entomocidus</i>	+	+	+	+	+	-	-	-	-	+	+	+
var. <i>entomocidus</i>												
<i>B. entomocidus</i>	+	+	+	+	+	-	-	-	-	w	w	-
var. <i>subtoxicus</i>												
G1	-	-	+	-	+	w	+	-	-	+	+	w
G2	+	-	-	-	+	-	+	-	-	+	+	+
<i>B. cereus</i> 634	+	+	+	+	-	-	-	-	-	w	w	-
<i>B. cereus</i> 826	+	+	+	+	-	-	+	-	-	+	+	+
<i>B. cereus</i> 827	-	-	-	-	-	w	+	-	-	+	+	+
<i>B. cereus</i> var. <i>mycoides</i> A.C.	-	+	+	+	-	-	+	-	-	+	+	+

w = weak reaction.

Table 2. *Plating efficiencies of phages A, B, C and D on the susceptible Bacillus strains*

Test organism	Phage			
	A	B	C	D
	Plating efficiency			
<i>B. entomocidus</i> var. <i>entomocidus</i>	100	50	150	200
<i>B. entomocidus</i> var. <i>subtoxicus</i>	500	100	180	200
G1	0	0	100	0
G2	5	0	0	0
<i>B. cereus</i> 634	100	5	1	1
<i>B. cereus</i> 826	100	5	100	100
<i>B. cereus</i> var. <i>mycoides</i> A.C.	0	0.5	0.1	0.3

Plaque morphologies

Plaque morphology showed considerable variation which was clearly dependent on both the phage and the test organism. Plaques ranged from completely clear areas about 1 mm. in diameter (e.g. phage A acting on *Bacillus entomocidus* var. *entomocidus*; phage B acting on the variety *subtoxicus*) to faintly discernible plaques in which there was no complete clearing of the lawn (e.g. most of the plaques formed on *B. cereus* 634 and on *B. cereus* var. *mycoides* A.C.). In general, phage D tended to produce smaller and less well-defined plaques than the others; phage A behaved characteristically in producing turbid plaques on lawns of *B. entomocidus* var. *subtoxicus*. The plaque morphologies were not sufficiently characteristic to be of taxonomic value.

Phage resistance

Colonies of phage-resistant organisms frequently developed in areas of confluent lysis resulting from the action of high phage concentrations. Some of these colonies

were picked and tested for sensitivity to the different phages and for ability to produce lecithinase, acetylmethylcarbinol and protein crystals. In some cases organisms resistant to one phage were treated with a second phage and doubly resistant variants isolated.

Resistance to phage B was frequently accompanied by the development of resistance to phages C and D but was not associated with resistance to phage A except in the case of *Bacillus entomocidus* var. *subtoxicus*. Selection for resistance to phage A did not normally yield strains resistant to the other phages, although results were not always clear cut and increased resistance was occasionally seen with several strains. It was possible to obtain organisms showing a wide variety of phage sensitivity patterns and by selection in several steps to derive strains completely resistant to the four phages from originally susceptible strains.

In all cases changed susceptibility to the phages had no effect on the abilities of the organisms to produce lecithinase or acetylmethylcarbinol. Neither were there any instances of gain or loss of ability to form crystals, but strains of *B. entomocidus* var. *subtoxicus*, which normally produce rather slender delicate bi-pyramidal crystals, produced larger fatter bi-pyramids when they developed resistance to phage B.

DISCUSSION

Apart from the detailed study of phages derived from *Bacillus cereus* strain W reported by McCloy (1951, 1958) and a brief note by Földes, Meretey & Varga (1961) our knowledge of phages in the *B. cereus* group is scant. Only one short communication concerns phages of the crystal-formers. Gochnauer (1960) isolated phages from lysogenic strains which were active against *B. entomocidus* var. *entomocidus* and against the 'terminalis' strain of *B. cereus* but he made no mention of lecithinase production by his strains. The 'terminalis' strain in my collection is strongly lecithinase positive and resistant to all four of my phages.

Many strains of *Bacillus cereus* carry temperate phage and mutation of such phage to a form capable of attacking the lysogenic host appears to be common so that spontaneous phage lysis is frequently seen in bacteria of this group. The most thorough investigation of this type of behaviour is that of McCloy (1951, 1958) who showed that a strain of *B. cereus* (strain W) regularly produced a phage ($W\alpha$) which formed plaques on strain W. Strain W was lysogenic with a temperate phage, $W\beta$, of which $W\alpha$ was a virulent mutant. A further non-lysogenizing phage, $W\gamma$, was also found in cultures of strain W. The three phages were identical in serological and other characters and McCloy (1958) studied the close relationship which exists between them. In the present work cultures of *B. entomocidus* var. *entomocidus* and of *B. cereus* 826 behaved like McCloy's strain W in that they regularly produced phages (A and D, respectively) which were able to produce plaques on the host strain. Spontaneous plaques appeared even when the cultures were derived from single spores and these bacteria presumably carry temperate phages and are susceptible to virulent variants of them.

Studies of *Bacillus cereus* phages show patterns of group specificity at subspecies level. Földes *et al.* (1961) isolated phages active against the streptomycin-resistant *B. cereus* strain 569 from soil and found them to be active only against certain *B. cereus* strains. The phages did not attack *B. anthracis* or strains of *B. cereus* which

produced the 'anthrax wall polysaccharide'. Autolysates of phage-resistant strains gave a precipitation reaction with anti-anthrax polysaccharide sera while autolysates of sensitive strains did not give this reaction. The *B. cereus* cell wall polysaccharide which precipitates anthrax antibody consists of galactose and glucosamine; the corresponding polysaccharide of phage-sensitive strains contains glucose, glucosamine and an unknown hexosamine. The phages described by McCloy (1951) attacked certain *B. cereus* strains and the anthrax bacillus. The group of phage susceptible organisms described in the present communication consists of strains with little or no ability to produce lecithinase and includes both crystal-forming and non-crystal-forming bacteria.

Heimpel & Angus (1958) suggested a scheme of classification of crystal-formers, recognizing parasporal body formation as an essential criterion for separating them from *Bacillus cereus* and *B. anthracis* and then further subdividing them into *B. thuringiensis* and *B. entomocidus* on a basis of acetylmethylcarbinol and lecithinase production. *B. finitimus* constitutes a third species in which the parasporal body does not separate from the spore at maturity. It produces both lecithinase and acetylmethylcarbinol and was not susceptible to any of my phages. The Heimpel & Angus scheme of classification is of considerable value since it brings order into the group but it does not accommodate lecithinase-negative strains of *B. cereus* (which vary in their abilities to produce acetylmethylcarbinol) or lecithinase-negative acetylmethylcarbinol-producing organisms such as *B. cereus* var. *mycoides* A.C. and crystal-former G2. It is generally accepted that the crystal-formers are very similar to *B. cereus*. I have observed a common spore precipitinogen present in all the crystal-formers and *B. cereus* strains in my collection. The close relationship between crystal-formers and non-crystal-formers is further supported by the present findings in which phage sensitivity cuts across several of the characters used for subdivision in these organisms.

There seem to be two types of '*Bacillus cereus*': one group of strains which produces large amounts of lecithinase and another group which does not do so. Both groups contain strains with the typical rhizoid colonial morphology of the mycoides variety and both groups contain crystal-forming strains, many of which are pathogenic for lepidopterous larvae. The characters discussed above could be used as the basis for a scheme of classification of these organisms. But it is clear that our knowledge of the group is still fragmentary; the present observations may perhaps serve better as a stimulus to further work than as the basis for a taxonomic concept of doubtful validity.

Much remains to be learned about the ecology of crystal-formers. Toumanoff (1960) showed that the disease 'flacherie' caused by a crystal-former in the silkworm *Bombyx mori* can be transmitted from one generation of larvae to the next. My own closed colony of *Galleria mellonella* is normally free from visible disease but crystal-forming bacteria (strains G1, G2) have been isolated from diseased larvae on occasions separated by a year during which there were no signs of disease. Further isolations of organism G2 have been made from excreta of the colony but infected larvae have remained rare. Such observations emphasize the complexity of the host/parasite relationship. The finding that the two organisms G1 and G2 have a different pattern of phage susceptibility suggests that phage typing might play an important part in its elucidation.

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Utilization of L-Glutamic and 2-Oxoglutaric Acid as Sole Sources of Carbon by *Escherichia coli*

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SUMMARY

A wild-type strain of *Escherichia coli* (W) did not utilize glutamate or oxoglutarate as sole source of carbon for growth, but mutants able to grow on each of these compounds were isolated. The abilities to utilize glutamate and oxoglutarate did not necessarily accompany each other. The evidence presented supports the view that both kinds of mutant involve changes in a permeation mechanism. The mutation to growth on glutamate was always accompanied by appearance of sensitivity to inhibition by 2-methyl-DL-glutamic acid and by partial or even complete loss of glutamic acid decarboxylase. It is proposed that the permeation mechanism for glutamate also allows entry of 2-methylglutamate, a compound which prevents glutamine formation. The loss of glutamate decarboxylase remains unexplained.

INTRODUCTION

In the course of studies on the mechanism of utilization of ammonia by cultures of *Escherichia coli* it was noted that various strains differed in their ability to utilize L-glutamate and 2-oxoglutarate. Some of the strains used, including strain W, were unable to grow on minimal media in which either of these compounds served as the carbon source (Halpern & Umbarger, 1960). Later, mutants capable of utilizing glutamate or oxoglutarate were selected. The acquisition of the ability to grow on one of the two compounds appeared to be independent of the ability to grow on the other. It was of interest that all the wild-type *E. coli* strains which were unable to grow on glutamate possessed a highly active L-glutamic acid decarboxylase, while those that could utilize glutamate for growth showed no such activity. Similarly, mutants selected for ability to grow on glutamate lost most or all of their decarboxylase activity. Whereas no direct causal relationship between growth on glutamate and the absence of glutamic acid decarboxylase could be demonstrated, evidence has

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been obtained which implicates specific permeation systems as prerequisites for the utilization of glutamate and oxoglutarate. These experiments are described in this paper. A preliminary report has been given elsewhere (Halpern & Umbarger, 1959).

METHODS

Micro-organisms. The organisms used in this work were: *Escherichia coli* strains W, K-12, B and H; *Aerobacter aerogenes* strain 1033; a strain of *Pseudomonas aeruginosa* from the stock culture collection of the Department of Bacteriology and Immunology, Harvard Medical School.

Media. The minimal medium of Davis & Mingioli (1950), with the omission of citrate, was used throughout. The various compounds used as carbon sources were prepared in separate solutions and added aseptically to the basal medium after autoclaving.

Selection of mutants. *E. coli* strain W was inoculated (about 5×10^7 organisms/ml.) into 100 ml. amounts of minimal medium with 0.5% (w/v) L-glutamic or 2-oxoglutaric acid as the carbon source. The cultures were incubated at 37° with shaking. Usually there was no detectable growth even after prolonged incubation (72 hr.). However, rapid increase in optical density ensued on the 5th day of incubation, the final concentrations of organisms equalling those obtained with succinate as carbon source. Such cultures were plated on eosin + methylene blue agar and single colonies were picked and tested for their ability to grow on the respective carbon source. Three glutamate-utilizing mutants were obtained on separate occasions by this method, and were designated W/Gl₁, W/Gl₂ and W/Gl₃. One mutant utilizing oxoglutarate was also obtained and was designated W/KG. From strain W/Gl₁ a secondary mutant was later selected for ability to utilize oxoglutarate in addition to glutamate; this mutant was designated *E. coli* W/Gl₁/KG. Similarly, two glutamate-utilizing mutants of *E. coli* H were obtained using this method, designated H/Gl₁ and H/Gl₂.

Growth experiments. For the determination of growth rates inocula from log-phase cultures were transferred to 20 ml. of appropriate medium in 250 ml. Erlenmeyer flasks with cuvette side arms. Such cultures were incubated at 37° in a New Brunswick gyratory water-bath shaker. The changes in optical density were followed in a Klett-Summerson photoelectric colorimeter with a No. 42 filter.

Oxygen uptake measurements. Conventional manometric techniques were employed for the determination of oxygen uptake by cell suspensions.

Reduction of triphenyltetrazolium chloride. The capacity of bacterial suspensions, and bacteria disintegrated by sonic oscillation (15 ml. of a cell suspension of $OD \times 10 = 60$ at 550 m μ in M/15 phosphate buffer, pH 7.5 was treated for 8 min. in a Raytheon 10 KC magnetostriuctive sonic oscillator), to reduce triphenyltetrazolium chloride in the presence of glutamate was determined under both aerobic and anaerobic conditions. For the anaerobic experiments Thunberg tubes were used. The reaction was stopped by the addition of 0.1 ml. 4 N-HCl. The formazan formed was extracted with 2 ml. isobutanol and the colour intensity measured at 485 m μ in a Coleman Junior spectrophotometer.

Preparation of cell-free extracts. Cell-free extracts used in the determinations of the enzymic activities studied were prepared by disintegration of bacterial suspen-

sions ($OD \times 10 = 60$ at $550 m\mu$) in a Raytheon 10 KC magnetostrictive sonic oscillator in the cold for 8 min. The cell debris was removed by centrifugation in the cold ($4-6^\circ$) for 15 min. at 28,000 g. The protein content of the supernatant liquid was determined by Mehl's biuret method (Mehl, 1945).

Assay of glutamic acid dehydrogenase. Glutamic acid dehydrogenase activity of cell-free extracts was determined by reduction of triphosphopyridine nucleotide (TPN) in the presence of L-glutamate, and its rate was followed at $340 m\mu$ in a Beckman model DU spectrophotometer. The reaction mixture contained: bacterial protein, 0.3 mg., different concentrations of L-glutamate (pH 8.7), TPN, $0.3 \mu\text{mole}$, phosphate buffer (pH 8.7) $120 \mu\text{mole}$, in a total volume of 3 ml. Increase in absorption at $340 m\mu$ was followed for 5 min. at room temperature. Enzyme activity was calculated from the reaction rate for 1 min. between the initial 30 and 90 sec. after addition of glutamate.

Assay of glutamine synthetase. Glutamine synthetase activity of cell-free extracts was determined in a system similar to that described by Fry (1955). The amounts of glutamine formed and glutamic acid utilized were estimated after paper chromatography, according to Giri, Radhakrishnan & Vaidyanathan (1952).

Assay of glutamic acid decarboxylase. Glutamic acid decarboxylase activity of extracts of cells grown in the presence of glutamate was determined manometrically or by paper chromatography as previously described (Halpern & Grossowicz, 1956).

*Determination of ^{14}C -L-glutamate uptake by logarithmic phase cultures of *Escherichia coli* strains W and W/Gl₁.* *E. coli* W and W/Gl₁ were grown overnight (inoculum about 10^7 organisms/ml.) in 20 ml. minimal medium containing a limiting amount of glucose (0.05%, w/v). Logarithmic growth was immediately resumed upon addition of 0.3% (w/v) glucose. When the cultures reached a turbidity of 0.36 (at $550 m\mu$), 1.5 ml. samples were withdrawn and incubated with various concentrations of ^{14}C -uniformly labelled L-glutamate (The Radiochemical Centre, Amersham, Bucks), in the presence and in the absence of 2-methyl-DL-glutamic acid, $2.79 \times 10^{-1}\text{M}$, for 4 min. at 37° in a total volume of 2 ml. The reaction mixture was filtered in the cold through a bacterial membrane filter, 25 mm. in diameter (Membranfilter, Göttingen, Gruppe:3) and rinsed with about 80 ml. ice-cold water. The filters were air-dried and their radioactivity measured in a Geiger-Muller counter (Tracerlab, Inc.).

RESULTS

Growth studies

Following the observation that growth of *Escherichia coli* strain W on glutamate or on 2-oxoglutarate was possible only following selection of variants from a large population, experiments were performed to compare the growth rates of the variants with that of the wild strain on each of several carbon sources. Table 1 gives the results of such experiments. It was noted that with the acquisition of the ability to utilize glutamate or 2-oxoglutarate, there was little or no change in the growth rate on glucose or on another tricarboxylic acid cycle intermediate, succinate. In addition, it was apparent that the ability to grow on either glutamate or 2-oxoglutarate had no effect on the ability to grow on the other. Furthermore, for those strains which were able to grow on them, succinate, glutamate or 2-oxoglutarate were utilized for growth about equally effectively. Although the wild-type parent utilized glutamate as the source of nitrogen, it did so only poorly (column 6, Table 1).

However, the strain which utilized glutamate as a source of carbon (strain W/GI₁) also used it better as a source of nitrogen, although it was able to utilize ammonia more effectively as a nitrogen source (column 2, Table 1).

Table 1. *Growth of Escherichia coli strain W and its mutants on different compounds serving as a sole source of carbon*

The experiment was performed in minimal medium, to which 0.5% (w/v) of the respective carbon source was added. The inocula were prepared from overnight cultures on succinate. The initial concentrations were about 2×10^8 organisms/ml. For other conditions, see Methods.

Strain	Carbon source				
	Glucose	Succinate	L-glutamate	2-oxo-glutarate	Glucose + glutamate ((NH ₄) ₂ SO ₄ omitted)
	Generation time (hr.)*				
<i>E. coli</i> W	1.00	1.50	> 6	> 16	3.00
<i>E. coli</i> W/KG	1.00	1.50	> 7	1.30	—
<i>E. coli</i> W/GI ₁	1.25	1.65	1.55	> 16	1.70
<i>E. coli</i> W/GI ₁ /KG	—	—	1.55	1.40	—

* Time required for doubling of the optical density of the culture in the logarithmic phase of growth.

Table 2. *Rates of oxygen uptake by suspensions of Escherichia coli W and its mutants on various substrates*

The bacteria were grown overnight on succinate as the carbon source, washed and re-suspended in 0.05 M phosphate buffer (pH 8.7). Each Warburg vessel contained about 15 mg. equiv. dry weight organism, MgCl₂, 10 μ mole, and phosphate buffer (pH 8.7) 50 μ mole in the main compartment; 10 μ mole of substrate in the side arm, and KOH 40% (w/v) 0.2 ml., in the centre well; total volume 1.5 ml.; incubation at 30°.

Organisms	Substrate		
	Succinate Q_{O_2} *	L-Glutamate Q_{O_2} *	2-Oxoglutarate Q_{O_2} *
<i>E. coli</i> W	540	49 (93)†	90
<i>E. coli</i> W/KG	540	8	264
<i>E. coli</i> W/GI ₁	522	402 (18)†	100
<i>E. coli</i> W/GI ₁ /KG	468	336	234

* The values obtained for endogenous respiration were subtracted from those obtained in the presence of substrate.

† Numbers in parentheses represent the amount of glutamic acid recovered at the end of the experiment, as % of initial value.

Rates of oxygen uptake

Experiments were performed to determine the oxygen uptake of washed suspensions of the different strains. There was a very good correlation between respiratory activity towards the different carbon sources and the ability of a given strain to utilize these substances for growth (Table 2). When glutamate was the substrate, over 80% of it disappeared in the presence of strain W/GI₁, whereas in the presence of the wild-type organism practically all of the added glutamate remained at the end of the experiment.

Glutamic acid dehydrogenase activity

Since glutamic acid dehydrogenase might be involved in the early steps of glutamic and 2-oxoglutaric acid metabolism, the activity of this enzyme was investigated in extracts of *Escherichia coli* W and of the glutamate and oxoglutarate utilizing mutants. No significant differences between the activities of the various strains were detected.

Reduction of triphenyltetrazolium chloride

Different results were obtained when suspensions of *Escherichia coli* strains W and W/Gl₁ were tested for their ability to catalyse the transfer of electrons from glutamate to triphenyltetrazolium chloride. It can be seen that with intact organisms (Fig. 1) the apparent affinity of the wild strain for glutamate was much

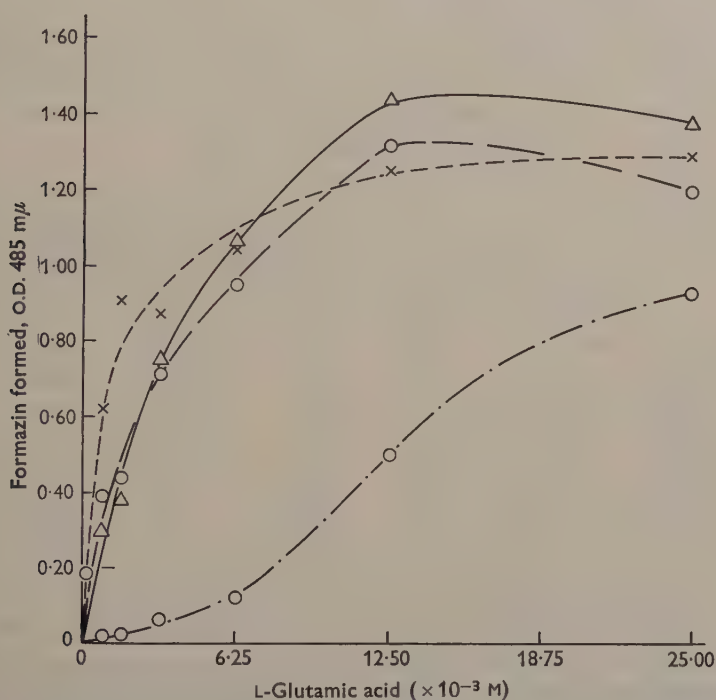


Fig. 1. Reduction of triphenyltetrazolium chloride by whole cell suspensions and by sonically disintegrated cells of *E. coli* W and W/Gl₁ with glutamate as the electron donor. Overnight cultures of *E. coli* W and W/Gl₁ on minimal medium, containing 1% (w/v) Na-succinate and 0.5% (w/v) L-glutamic acid, were washed and resuspended in M/15 phosphate buffer, pH 7.5. Both suspensions were adjusted to the same optical density ($OD \times 10 = 60$ at 550 m μ). Part of each suspension (15 ml.) was disrupted in a Raytheon 10 KC magnetostrictive sonic oscillator for 8 min., and the remaining part was diluted with an equal volume of M/15 phosphate buffer, pH 7.5. The reaction mixtures contained: whole cell suspension (0.945 mg. dry weight of bacteria) or sonically disintegrated cells (4.413 mg. dry weight of bacteria), L-glutamic acid as indicated, triphenyltetrazolium chloride, 0.06% (w/v), phosphate buffer pH 7.5, 467 μ moles, in a total volume of 1.6 ml.; incubated aerobically at 37° for 10 min. The reaction was stopped by addition of 0.1 ml. 4 N-HCl; the formazan formed was extracted with isobutyl alcohol, 2 ml., and read at 485 m μ . ---○---, W whole cells; —○—, W broken cells; ---×---, Gl₁ whole cells; —△—, Gl₁ broken cells.

lower than that of the Gl_1 mutant. In contrast, when sonically disrupted suspensions were used, the differences in the reaction rates of the two strains at either low or higher concentrations of glutamate were slight and perhaps not significant.

Effect of 2-methyl-DL-glutamic acid

Further evidence on the nature of the mutation to glutamate utilization was obtained by using a glutamate analogue, 2-methyl-DL-glutamate. This compound has been shown (Ayengar & Roberts, 1952) to inhibit the growth of certain lactic acid bacteria, presumably by inhibiting the conversion of glutamate to glutamine. As shown in Table 3, when 2-methylglutamate was added to a glucose minimal medium, those *Escherichia coli* strains able to use glutamate as sole carbon source (W/Gl_1 , W/Gl_2 , B, H/ Gl_1) were inhibited by the analogue. Strains unable to grow on glutamate (W, K-12, H) were resistant.

The correlation suggested by the results shown in Table 3 was further supported by isolating from the glutamate-utilizing mutant strain W/Gl_1 a variant, strain

Table 3. *Inhibition of growth of different Escherichia coli strains by 2-methyl-DL-glutamic acid*

For conditions of growth see Methods.

Strain	No additions (control) Klett reading	In the presence of 2-methyl-DL-glutamic acid			
		0.15 % (w/v)		0.50 % (w/v)	
		Klett reading	Inhibition (%)	Klett reading	Inhibition (%)
<i>E. coli</i> W	375	313	16.5	290	22.7
<i>E. coli</i> W/Gl_1	443	Not tested		26	94.1
<i>E. coli</i> W/Gl_2	242	31	87.2	5	97.9
<i>E. coli</i> K-12	280	345	0	Not tested	
<i>E. coli</i> B	325	23	92.9	10	96.9
<i>E. coli</i> H	415	275	33.7	202	51.3
<i>E. coli</i> H/ Gl_1	510	12	97.6	15	97.1

Table 4. *Uptake of ^{14}C -L-glutamate by Escherichia coli strains W and W/Gl_1 in the presence and in the absence of 2-methyl-DL-glutamic acid*

The reaction mixture contained: logarithmic phase organisms equiv. 0.567 mg. dry wt. bacteria, in minimal medium containing 0.3 % (w/v) glucose, ^{14}C -L-glutamate (uniformly labelled, 107 c.p.m./ μ mole) and 2-methyl-DL-glutamic acid as indicated, in a total volume of 2 ml.; incubation in a water-bath with shaking (about 200 oscillations/min.) at 37° for 4 min. For other conditions see Methods.

^{14}C -L-glutamate added (M)	Uptake of ^{14}C -L-glutamate (c.p.m.)					
	<i>E. coli</i> W			<i>E. coli</i> W/Gl_1		
	2-Methyl-DL-glutamate added			2-Methyl-DL-glutamate added		
	None	$2.79 \times 10^{-4}M$	% Inhibition	None	$2.79 \times 10^{-4}M$	% Inhibition
9.3×10^{-8}	287	230	19.9	455	225	50.1
2.8×10^{-5}	631	523	17.1	1024	491	52.0
8.4×10^{-5}	1070	992	7.3	1703	1251	26.5

W/G₁/MG, selected for ability to grow in glucose minimal medium + 2-methylglutamate. This variant was found to have lost its ability to grow on glutamate. Similarly, another derivative, strain W/G₁-8, was selected by means of the penicillin selection method (Davis, 1948) for restoration of the original inability to grow on glutamate; this strain was found to have lost its sensitivity to 2-methylglutamate. In contrast, when the glutamine-synthesizing systems were tested in extracts it was observed that the activity in extracts of both *Escherichia coli* strains W and W/G₁ was inhibited by the analogue (78–88 % inhibition by 0.011 M-2-methylglutamate).

Further evidence which stresses the relationship between the ability of the organism to utilize L-glutamate and the inhibitory effect of 2-methyl-DL-glutamate on its growth was obtained by uptake experiments described in Table 4. As shown in Table 4, uptake of ¹⁴C-labelled L-glutamic acid by *Escherichia coli* W/G₁ was considerably higher than that by the W strain and was inhibited to the extent of 50 % in the presence of the 2-methyl analogue. On the other hand, addition of 2-methyl-DL-glutamic acid caused an inhibition of L-glutamate uptake by strain W of less than 20 %. The residual (uninhibited) uptake was virtually the same in both strains.

Table 5. Comparison of L-glutamic acid decarboxylase activities of various mutants of *Escherichia coli*

The reaction mixtures in Warburg flasks contained: bacterial extract equiv. 0.75 mg. protein, L-glutamic acid, 15 μ mole; acetate buffer (pH 4.5) 90 μ mole; pyridoxal phosphate, 150 μ g; in a total volume of 1.5 ml. Incubation was at 30°. The extract in each case was prepared from organisms harvested in the logarithmic phase from a medium containing initially 1.0 % (w/v) glucose and 0.5 % (w/v) L-glutamate.

Strain	Glutamate as sole carbon source	Q _{O₂}	Strain	Glutamate as sole carbon source	Q _{O₂}
<i>E. coli</i> W	—	592	<i>E. coli</i> H/G ₁	+	0
<i>E. coli</i> W/G ₁	+	20	<i>E. coli</i> K-12	—	200
<i>E. coli</i> W/G ₁ ₂	+	224	<i>E. coli</i> B	+	0
<i>E. coli</i> W/G ₁ ₃	+	20	<i>A. aerogenes</i> 1033	+	0
<i>E. coli</i> H	—	1467	<i>Ps. aeruginosa</i>	+	0
<i>E. coli</i> H/G ₁	+	452			

Glutamate decarboxylase activity in glutamate-utilizing strains

The only enzymic difference noted between extracts of organisms unable to grow on glutamate and extracts of those able to utilize glutamate was in the decarboxylation of glutamate. Thus *Escherichia coli* strains W, K-12 and H exhibited strong glutamic acid decarboxylase activity, whereas variants of these strains which can grow on glutamate, showed either a partial or a complete loss of the decarboxylase. It was also found that the three wild-type organisms which were observed to utilize glutamate as a carbon source, *E. coli* strain B, *Aerobacter aerogenes* strain 1033 and the strain of *Pseudomonas aeruginosa*, exhibited no glutamic acid decarboxylase activity (Table 5). At present, the mechanism underlying this apparent correlation is not clear. The relationship, however, does not appear to be a direct one. For example, selection for loss of the ability to utilize glutamate or for resistance to 2-methylglutamate (such as strains W/G₁-8 and W/G₁/MG described above) did not select organisms which had gained the ability to decarboxylate glutamate.

DISCUSSION

The difference between *Escherichia coli* W and its mutants, with respect to their ability to utilize glutamate and oxoglutarate for growth, might have been due either to changes in the respective metabolic pathways or to changes in the ability of the organisms to take up the substrates. Although glutamate oxidation by strains selected for growth on glutamate was much higher than that in strains unable to grow on glutamate, the glutamic dehydrogenase activities of the extracts prepared from the two kinds of strains were not significantly different. This difference must be accounted for, therefore, by a difference in the ease of entry of glutamate into the cells of the two strains. This hypothesis is directly supported by the observations on the difference in tetrazolium reduction as between whole organisms and extracts. Thus, at low concentrations of glutamate there was a distinct difference in the rates of tetrazolium reduction by intact organisms of the two strains. This difference could be essentially abolished by disrupting the organisms or by using higher concentrations of glutamate. The change in the glutamate-utilizing mutant could probably best be explained by an increased capacity to transport glutamate into the cell.

A difference between the penetration of glutamate into organisms of strain W and into organisms of strain W/Gl₁ could also explain the difference in the sensitivity of the two kinds of organism to 2-methylglutamate, a compound which inhibits glutamine formation by extracts of both strains. It would appear that the same mechanism which is responsible for the transport of glutamate into the cell also facilitates the penetration of the analogue.

Although it is not clear how the loss of decarboxylase activity is related to the utilization of glutamate, the two phenomena nevertheless seem to be connected. In every mutant examined the appearance of the ability to grow on glutamate was accompanied by a total or at least partial loss of glutamic decarboxylase activity. Even the relatively high decarboxylase activity of strain H/Gl₁ ($Q_{CO_2} = 452$) comprises only 30 % of the activity of the parent H strain. The same argument holds also for the decarboxylase of strain W/Gl₂, which, although being as active as that of *Escherichia coli* K-12 unable to grow on glutamate, is nevertheless only one-third as active as that of its parent W strain.

Finally, it may be pointed out that the data in Table 1 provide evidence that the mutation to growth on 2-oxoglutarate was also due to a change in a penetration mechanism. The present evidence for a similar system in *Escherichia coli* is less conclusive than in the case of glutamate. However, if it may be assumed that glutamate utilization proceeds via 2-oxoglutarate, it is clear that an organism which metabolizes glutamate at a rate sufficient for growth must also metabolize endogenous oxoglutarate at a rate sufficient for growth. Since such strains do not grow on exogenous oxoglutarate, their failure to do so must be attributed to the inability of this compound to enter the cell. It is of interest that Kogut & Podoski (1953) presented evidence for the existence of an oxoglutarate permeation system in a *Pseudomonas* strain.

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The Cultural and Physiological Characters of the *Pediococci*

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SUMMARY

The cultural and physiological characters of 89 strains of *pediococci* have been studied. Proposals are made for extension of the genus and its subdivision into two and possibly three groups. The reactions of eleven strains of *Aerococcus viridans* were also investigated.

INTRODUCTION

Organisms described as *pediococci* have been studied for some considerable time, mainly in relation to problems in the brewing industry (Balcke, 1884; Mees, 1934; Shimwell & Kirkpatrick, 1939). More recently these organisms have been found in appreciable numbers in fermenting vegetable material (Pederson, 1929; Pederson & Albury, 1950; Pederson, Albury & Breed, 1954), in 'caecal faeces' of turkeys (Harrison & Hansen, 1950), in the rumen of cows (Bauman & Foster, 1956), in summer sausage (Deibel & Niven, 1957), and in cheese (Naylor & Sharpe, 1958; Dacre, 1958*a, b*).

Systematic studies of the physiological characters (Pederson, 1949; Felton & Niven, 1953; Jensen & Seeley, 1954; Pederson *et al.* 1954) and of the nutritional requirements (Jensen & Seeley, 1954) have led to recognition of the *pediococci* as belonging to a separate genus and to its classification within the family *Lactobacillaceae* in the tribe *Streptococceae*. This view has been incorporated in the seventh edition of *Bergey's Manual* (1957), where *Pediococcus* is described as a genus of Gram-positive cocci occurring singly and in tetrads, pairs and short chains, micro-aerophilic, generally catalase-negative, homofermentative, producing optically inactive lactic acid from carbohydrates, producing acidity and cloudiness in beer, and found as saprophytes in fermenting vegetable juices. Within the genus thus described two species only are recognized, *P. cerevisiae* Balcke, 1884, and *P. acidilactici* Lindner, 1887, distinguished by their optimum growth temperatures and ability to grow in beer.

In the course of a study of bacterial changes occurring during the ensilage process (Hoffman, Wolf & Barker, 1957) a large number of Gram-positive cocci was isolated which resembled the *pediococci* in certain characters. However, some of the silage isolates differed sufficiently from the descriptions of *pediococci* to suggest that the genus might be wider than previously thought. We have therefore made a survey of the cultural, physiological and serological characters of strains from a wide range of sources. Such an investigation seems to be particularly appropriate at the present time since the possibility of utilizing *pediococci* in fermentation processes has recently been proposed (Pederson & Albury, 1950; Dacre, 1958*a*; Dr C. F. Niven

Jun., personal communication). A possible relationship between the pediococci and members of the genus *Aerococcus* (Williams, Hirsch & Cowan, 1953) was suggested by Jensen & Seeley (1954) and by Dr Ellen I. Garvie (personal communication). We have therefore included in the survey some *Aerococcus* strains.

METHODS

Sources of cultures. Eighty-nine isolates (including 39 new isolates from silage) were collected; 31 of these were representatives of named species. Most of the experimental work was carried out with all isolates; where this was not practicable isolates considered to be representative were selected for investigation.

In addition to the pediococcus cultures, 11 isolates of *Aerococcus viridans* were studied. A list of the isolates is given in Table 1, together with the references for the named species.

Maintenance of stock cultures and methods of cultivation. For maintenance of stock cultures, preparation of inocula and in all experimental work, 'Oxoid' tomato juice (TJ) broth or tomato juice (TJ) agar, adjusted to pH 6.6, were used unless otherwise stated. The following were exceptions to this rule: for strain Tc. 1 sodium chloride (5 %, w/v) was added to the medium; and for the aerococci glucose Lemco broth (Shattock & Hirsch, 1947) or glucose yeast extract (GY) agar (containing, as %, w/v; peptone, 1.0; Yeastrel, 0.3; glucose, 1.0; NaCl, 0.25; agar, 1.0; at pH 7.4) was used.

Cultures were incubated aerobically except where otherwise stated. The normal incubation temperature was 30° except for isolates M-1, 8519 and 8520, for which it was 22°.

Stock cultures were maintained as stab cultures and stored at 4°. New transfers were made at 3-monthly intervals.

For use as inocula, vigorously growing cultures were obtained by making at least three successive subcultures. The incubation period was 24 hr. except for strains M-1, 8519, 8520 and Tc. 1 which required 72 hr. A 'standard' inoculum consisted of one loopful (about 4 mm. diameter) of such a vigorously growing culture/5 ml. test medium, and was used except where otherwise stated.

Morphology and staining reactions. The shape, arrangement and size of individual organisms were determined in Gram-stained smears prepared from 24 hr. cultures fixed by heat in the usual way. The Gram reaction (Jensen's modification; Mackie & McCartney, 1953) of 24 and 48 hr. liquid cultures was determined. Hanging drop preparations of 24 hr. broth cultures were used to determine motility. Smears from 72 hr. agar cultures were examined for the presence of spores according to Fleming's method (Mackie & McCartney, 1953) and Conklin's modification of Wirtz's method (Conklin, 1934). Muir's technique (Tanner, 1948) with 24 hr. agar cultures was used to search for capsules.

Cultural characters. The kind of surface colony was noted after incubation for 72 hr. and the form of growth in agar stab cultures observed after 48 or 96 hr. where necessary. Growth characters in liquid cultures were observed after incubation for 48 or 72 hr. Ability to form mucoid colonies on media containing sucrose was tested on the appropriate agar medium to which Seitz-filtered sucrose had been added to a final concentration of 5 % (w/v).

Conditions affecting growth

Except where otherwise stated, all results of experiments to determine the conditions which affected growth were recorded after 24, 48 hr. and 7 days of incubation.

Table 1. *Code number, species name, habitat and sources of cultures*

Code numbers	Species name	Habitat	Source
F-166, E-66, N-82, K-64, K-106, B-168	<i>P. cerevisiae</i> (Pederson, 1949)	Fermenting vegetable	(1)
A-1, C-1	<i>P. cerevisiae</i> (Pederson, 1949)	Spaghetti sauce	(1)
2-170, 3-124, 3-129, 4-60, 4-89, 5-51, 5-61, 6-107, 6-159, 6-163, D-32, D-95, D-118, N-91	<i>P. cerevisiae</i> (Pederson, 1949)	Fermenting vegetable	(2)
FP-1, FP-6	<i>P. cerevisiae</i> (Pederson, 1949)	Summer sausage	(3)
P-60	<i>P. cerevisiae</i> (Garvie, 1959)	Unknown	NCDO
P.c.	<i>P. pentosaceus</i> (Mees, 1934)	Unknown	(4)
M-1	<i>P. damnosus</i> (Claussen, 1903)	Beer	(4)
8519, 8520	<i>P. damnosus</i> var. <i>salicinaceus</i> (Mees, 1934)	Beer	NCIB
Tc.1	<i>P. halophilus</i> (Mees, 1934)	Unknown	(4)
PUE	<i>P. urinae equi</i> (Mees, 1934)	Horse urine	(4)
EJ-1	—	Fermenting cabbage	(2)
M-31	—	Milk	—
A-140, A-181, B-137, B-190	—	Cheese	—
SS-50, SS-61	—	Air of dairy	—
SS-69, SS-101, SS-128	—	Air of cowshed	(5)
HY-22s	—	Hay	—
BP-1, BP-2	—	Saliva	—
559 (strain A2, Dacre, 1958a)	—	Cheese	NCDO
C-1, C-2, C-6, C-14	—	Silage	(6)
P-45, P-128, S-18, S-180, S-182, S-188, S-190, S-191, S-290, S-333, S-334, S-336, S-338, S-339, S-340, S-342, S-344, S-447, S-524, S-525, S-526, S-527, S-532, S-533, L-16, L-20, L-22, L-24, L-92, L-95, L-148, L-171, L-223, L-345, L-347, L-351, L-352, L-354	—	Silage	—
7592, 7595, 7597, 7598, 7599, 7601, 7602	<i>Aerococcus viridans</i> (Williams, Hirsch & Cowan, 1953)	Air of occupied rooms	NCTC
7764, 7765, 7766, 7767		Milking machines	

(1) Professor C. S. Pederson, N.Y. State Agriculture Experiment Station, Geneva, N.Y., U.S.A.

(2) Professor H. W. Seeley, Cornell University, Ithaca, New York, U.S.A.

(3) Dr C. F. Niven Jun., University of Chicago, Illinois, U.S.A.

(4) Technische Hoogeschool, Delft, Holland.

(5) Dr M. E. Sharpe, National Institute for Research in Dairying, Shinfield, near Reading.

(6) Dr T. Gibson, The Edinburgh and East of Scotland College of Agriculture, Edinburgh.

NCIB = National Collection of Industrial Bacteria, Torry Research Station, Aberdeen.

NCDO = National Collection of Dairy Organisms, Shinfield, near Reading.

NCTC = National Collection of Type Cultures, London.

Oxygen requirement. Duplicate broth cultures were incubated aerobically, and anaerobically in an atmosphere of 95 % (v/v) hydrogen + 5 % (v/v) carbon dioxide. Visual estimation of growth was made after incubation for 24 or 72 hr. for slow growing strains.

Growth temperatures. To find the optimum growth temperature the amount of

growth after incubation for 24 hr. at 22°, 30° and 37° was estimated visually. With slow growing strains the results were read after 72 hr. of incubation. To indicate the range of growth temperatures, cultures were incubated at 10°, 40° and 45° in water baths controlled to within $\pm 1^\circ$.

Growth at pH 9.0 and pH 4.2. In these experiments the technique was based on that described by Shattock & Hirsch (1947) for testing growth of streptococci at pH 9.6. The following modifications were made: tomato juice (TJ) broth was substituted for glucose Lemco broth; to obtain the medium at pH 9.0 suitable quantities of the 0.1M-glycine buffer recommended by Shattock & Hirsch (1947) were added; for the medium at pH 4.2, sodium acetate + acetic acid buffer (Clark, 1928) at 0.04M was selected, since some inhibitory effects were noted at higher concentrations.

Tolerance to sodium chloride and Teepol. Ability to grow in 4 and 6.5% (w/v) sodium chloride and in 0.01, 0.05 or 0.1% Teepol was tested in TJ broth cultures.

Growth in wort, hopped wort and beer. The amount of growth was observed visually in wort, hopped wort and beer. The wort and hopped wort (about 6% hops) were obtained through the courtesy of Mr C. S. Everitt (Watney Mann Breweries, London) and the beer was commercially available bottled Carlsberg Lager. These media were sterilized by Seitz filtration.

Biochemical tests

Media used for biochemical tests were based on those commonly used for testing lactobacilli since optimal media for pediococci have not yet been devised. Where consistent with satisfactory results the lactobacillus media were simplified. Known positive and negative control cultures were included in each test series.

Catalase activity. Felton, Evans & Niven (1953) found that a medium of low carbohydrate content (YTG) gave a greater number of positive reactions than a medium of high carbohydrate content (APT). Gutekunst, Delwiche & Seeley (1957) recommended that cultures to be used for catalase tests should be neutralized after incubation. In the present work, preliminary tests were carried out with 12 isolates of pediococci to compare nutrient broth (containing (% w/v): Yeastrel, 0.3; peptone, 1.0; NaCl, 0.5; at pH 7.0) with TJ broth and GY broth as media for catalase tests. No qualitative differences were found but the reactions in nutrient broth were sometimes stronger. In view of this and of the recommendations of the above workers, nutrient broth was retained as the experimental medium. Twice the 'standard inoculum' (above) was used for 5 ml. medium and incubation was carried out for 24 hr., or 72 hr. when necessary. Two ml. of freshly prepared 3% (10 vol.) hydrogen peroxide were added and the cultures examined up to 30 min. for visible gas bubbles.

Haemolysis. Horse blood (5%, v/v) agar streak plates were prepared and incubated both aerobically and anaerobically. Pour plates were also made and incubated similarly. Results were read after incubation for 48 hr. and again after overnight storage at 4°.

Liquefaction of gelatin. Stab cultures were incubated at optimum temperature and examined for liquefaction after chilling at 7, 14 and 28 days. The nutrient gelatin medium had the same formula as the nutrient broth, with the addition of 14% (w/v) gelatin.

Reduction of nitrate. Incubation was carried out for 7 days in the medium of Davis (1955) from which salt solutions 'A' and 'B' had been omitted. Cultures were then tested for the presence of nitrite and of nitrogen gas as described in the *Manual for Pure Culture Study* (1954). The medium was tested for the presence of nitrite before incubation and for residual nitrate after incubation.

Production of ammonia from arginine. The method described by Niven, Smiley & Sherman (1942) was used.

Carbohydrate reactions. Yeast-extract peptone broth (containing, % w/v: peptone, 1.0; yeast extract, 0.5; NaCl, 0.5; MgSO₄, 0.05; MnSO₄, 0.05; at pH 7.0) was used as a basal medium for fermentation tests, and Seitz-filtered carbohydrate added to give 1 % (w/v) final concentration. Acid and gas production were determined after 7 days of incubation (indicator, 0.04 % (w/v) bromocresol purple, added after incubation), since preliminary results had shown that many isolates, especially fresh ones, were slow in producing acid. Acid once produced was not masked by subsequent production of alkaline substances. The carbohydrates tested were: arabinose, xylose, glucose, fructose, maltose, lactose, sucrose, trehalose, raffinose, inulin, dextrin, glycerol, mannitol, sorbitol, salicin.

Hydrolysis of aesculin. The method of Davis (1955) was used, except that Tween 80 (which according to Jensen & Seeley, 1954 is not required by *pediococci*) and salt solutions 'A' and 'B' were omitted from the medium, and sodium chloride (0.2 %, w/v), manganese sulphate (0.05 %, w/v) and magnesium sulphate (0.05 %, w/v) were added. The cultures were examined daily for 7 days.

Production of acetylmethylcarbinol from glucose and from lactose. Tests were carried out in the medium of Swartling (1951), modified in one series of experiments by the substitution of glucose for lactose. Cultures were incubated for 6 days and tested for acetylmethylcarbinol by Barritt's (1936) modification of the Voges-Proskauer test.

Final hydrogen ion concentration. Glucose (1 %, w/v) yeast-extract liquid cultures were incubated for 18 days and the final pH values measured electrometrically. Some isolates grew poorly in this medium but the use of tomato juice broth was considered inadvisable because of its natural content of reducing sugar which might have resulted in the production of acids from compounds other than glucose.

Production of carbon dioxide from glucose. The method of Gibson & Abd-el-Malek (1945) was used; cultures were examined daily for gas production during a 2-week incubation period.

Reaction in litmus milk. Litmus milk cultures were examined for reduction of indicator, change in pH value or coagulation during 28 days of incubation.

Type of lactic acid produced. The method of Pederson, Peterson & Fred (1926) was followed except that a continuous ether extraction apparatus was used, extracting the sample for 48 hr. The zinc content of the isolated zinc lactate was determined by the titrimetric method of Kolthoff & Sandell (1950) and the optical rotation determined polarimetrically, using the anhydrous salt in 1 % (w/v) aqueous solution. Six *pediococcus* strains were examined.

Utilization of ammonium salts as sole source of nitrogen. The medium and method described by Hucker (1924) were used. Incubation continued for 14 days.

Folic acid requirement. The method used was a modification of that outlined in the *Difco Manual* (1953). Tests were carried out in triplicate in 4 ml. amounts in

12 × 80 mm. EEL colorimeter tubes. Growth after 18 hr. of incubation was measured turbidimetrically with an EEL colorimeter. The folinic acid used in these experiments was supplied as 'leucovorin' by Lederle Laboratory Division Ltd. and was used in concentrations of 0, 0.15, 0.3 and 0.6 $\mu\text{g./ml.}$ Twenty isolates were examined for this requirement.

RESULTS

A comparison of named *pediococcus* cultures and unnamed strains showed certain features to be shared by all; these are regarded as characteristic of the genus. A number of additional features was possessed by some but not all isolates examined. On the basis of these additional features, two distinct physiological groups could be differentiated and there was some indication of a less well-defined third group. The results are presented in two sections: (i) characters common to all '*pediococci*'; (ii) characters used to differentiate the groups; the results are listed in Table 2. The reactions given by the aerococci are included for comparison. The criteria on which the subdivisions have been based are summarized in Table 3.

Characters common to all pediococci

Morphology and staining reaction. The organisms were spherical, occasionally ovoid, strongly Gram-positive, ranging in diameter from 0.36 to 1.43 μ . They were arranged in clusters, tetrads, pairs or singly, were non-motile, non-sporeforming and not encapsulated.

Cultural characters. Surface colonies were greyish-white, smooth, circular, low convex with entire margins. Growth in stab culture was beaded throughout the entire length of the stab, with a small amount of surface growth. Mucoid colonies were not formed on agar media containing sucrose.

Growth conditions. In the media described, growth was similar under aerobic and anaerobic conditions. The optimum temperature was 30° for all but three isolates; for these it was 22°. Growth was initiated at 10°. No strains were able to grow at pH 9.0. Wort provided a suitable substrate but the addition of hops exerted some inhibitory effect; no growth took place in the particular beer used.

Biochemical tests. A small zone of β haemolysis was produced on blood agar by a few isolates only. With the majority of isolates a zone of 'bleaching' similar to that described by Davis & Rogers (1939) for lactobacilli was noted. The organisms did not liquefy gelatin, did not reduce nitrate to nitrite or nitrogen gas, nor did they produce detectable amounts of carbon dioxide from glucose. Litmus milk was reduced, acidified and coagulated only rarely. The lactic acid produced by the six isolates tested was optically inactive. Small amounts of steam-volatile acids were produced in addition to lactic acid. No growth was observable in media in which ammonium salts constituted the sole source of nitrogen.

Characters on which was based differentiation into groups (Tables 2 and 3)

Group I. This group includes 38 isolates, of which 27 were cultures received as *Pediococcus cerevisiae*, one was received as *P. pentosaceus*, and the following were unnamed: C-1, C-2, C-6, EJ-1, SS-50, SS-128, BP-1, BP-2, P-45, P-128.

Members of this group were distinguished readily by: size of surface colonies on tomato juice agar, varying between 0.5 and 1.2 mm. in diameter; abundant growth

No. of strains	Peniococci					Aerococci				
	Group					Not grouped				
	<i>P. urinae</i> <i>equi</i> PUE		II a			II b			III	
	I	1	8	24	14	14	14	14	MI, 8519, 8520	<i>P. halophilus</i> Tc.1
Growth on TJB (pH 6.6)	+++	+++	++	+	++	++	++	++	3	11
Opt. temp. 22°	-	-	+	+	-	-	-	-	+	-
Opt. temp. 30°	+	+	+	+	+	+	+	+	+	+
Growth at 40°	+++	+++	++	+	++	+	+	+	+	+
Growth at 45°	+	+	+	+	+	+	+	+	+	+
Growth at pH 4.2	+	+	+	+	+	+	+	+	+	+
Growth in NaCl 4% (w/v)	+	+	+	+	+	+	+	+	+	+
Growth in NaCl 6.6%	+	+	+	+	+	+	+	+	+	+
Growth in Teepol 0.01%	+	+	+	+	+	+	+	+	+	+
Growth in Teepol 0.05%	+	+	+	+	+	+	+	+	+	+
Growth in Teepol 0.1%	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
NH ₃ from arginine	+	+	+	+	+	+	+	+	+	+
Acid from	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+	+	+
Inulin	+	+	+	+	+	+	+	+	+	+
Dextrin	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+	+	+
Litmus milk	+	+	+	+	+	+	+	+	+	+
Acid	+	+	+	+	+	+	+	+	+	+
Dye reduction	+	+	+	+	+	+	+	+	+	+
Coagulation	+	+	+	+	+	+	+	+	+	+
Hydrolysis of aesculin	+	+	+	+	+	+	+	+	+	+
AMC from glucose	+	+	+	+	+	+	+	+	+	+
AMC from lactose	+	+	+	+	+	+	+	+	+	+
Final pH in GYB after 18 days	3.7-3.9	5.0	3.9-5.8	3.9-6.4	4.4-5.0	4.4-5.0	4.4-5.0	4.4-5.0	4.9	4.6-5.1
Leucovorin requirement	+	+	+	+	+	+	+	+	+	+

+++ profuse growth; ++ good growth; + moderate growth.

Figures in brackets refer to number of strains giving indicated reaction when all strains were tested and not all gave identical reactions. When only a proportion of strains was tested the number positive/the number tested are given as a 'fraction'.

TJB = tomato juice broth; AMC = acetylmethylcarbinol; GYB = glucose yeast-extract broth.

in tomato juice broth; consistently low final pH value (3.7–3.9) in GY broth; vigorous growth at pH 4.2; production of ammonia from arginine. Thus, these isolates form a well-defined and easily recognizable group. In addition, 25 of these isolates were capable of growth at 45°, and 33 produced acetylmethylcarbinol from glucose and lactose. Catalase activity was demonstrated in 26 isolates. All isolates were tolerant of 4% (w/v) sodium chloride but a concentration of 6.5% delayed the growth of 27 isolates, to some degree. The addition of Teepol at 0.01 and 0.05% (v/v) did not affect growth but at 0.1% initiation of growth was delayed by 24 hr. for 3 isolates. Eight isolates in this group exhibited a specific requirement for leucovorin.

Group II. This group consisted of the cultures received as *Pediococcus halophilus* (Tc. 1) and the following 32 unnamed isolates: M-31, A-140, A-181, B-137, B-190, SS-61, SS-101, HY-22s, S-18, S-180, S-182, S-188, S-190, S-191, S-290, S-333, S-334, S-335, S-338, S-339, S-340, S-342, S-344, S-447, S-524, S-525, S-526, S-527, S-532, S-533, C-14, SS-69. Its members differed from group I in: producing surface colonies on tomato juice agar of only 0.3–0.4 mm. diameter; showing less vigorous growth in tomato juice broth; absence of catalase activity; failure to produce ammonia from arginine; no acetylmethylcarbinol from glucose or lactose. They were unable to grow at 45° or in 4% (w/v) NaCl within 24 hr. and grew at pH 4.2 only after prolonged incubation (up to 7 days). Two subdivisions were recognized within this group. The 24 isolates listed (S-18, SS-69), unlike the others, grew poorly in tomato juice broth, failed to grow at 40° within 24 hr. and generally did not produce acid from trehalose.

Table 3. *Main characters on which subdivision of pediococci may be based*

Species name	Group	Optimum growth temp.	Diameter of surface colonies on TJA (mm.)	Growth in TJB	Growth pH 4.2	Growth in 4% (w/v) NaCl		NH ₃ from arginine	AMC from glucose	Acid from dextrin
						24 hr.	48 hr.			
<i>P. cerevisiae</i>	1	30°	0.6–1.2	+++	+++	+++	+++	+	+	—
									almost always	
<i>P. 'parvulus'</i>	11	30°	0.3–0.4	+	Late	—	±	—	—	—
	111	30°	0.6–1.0	++	—	++	++	—	—	+
<i>P. damnosus?</i>		22°	0.3–0.6	Late	—			—	+	—
<i>P. halophilus?</i>		30°	0.6–0.8	Late	—	5% essential	—	—	—	—

TJA = tomato juice agar; TJB = tomato juice broth; AMC = acetylmethylcarbinol.
+++ Growth abundant; ++ good growth; + moderate growth.

Possible group III. This group was less well defined but is tentatively suggested for the following 14 unnamed isolates: L-16, L-20, L-22, L-24, L-92, L-95, L-148, L-171, L-223, L-345, L-347, L-351, L-352, L-354. These isolates resembled group I in size of surface colonies on tomato juice agar and abundant growth in tomato juice broth but could be differentiated by their inability to grow at pH 4.2 even when incubated for 7 days and by their failure to produce ammonia from arginine. They differed also from the majority of group I isolates in: failure to grow at 45°; absence of catalase activity; failure to produce acetylmethylcarbinol from glucose or lactose. They could be separated from group II isolates by: colony size; absence

of growth at pH 4.2; ability to grow in media containing 4% (w/v) NaCl within 24 hr.; acid production from dextrin.

Other isolates. Some isolates did not fall readily into any of the above three groups. *Pediococcus urinae equi* was indistinguishable in cultural characteristics from members of group I but differed from the majority of those in failure to grow at pH 4.2, inability to produce ammonia from arginine and acetylmethylcarbinol from glucose or lactose, higher final pH value in glucose broth (pH 5.0) and lower resistance to Teepol. It may be regarded as a member of a possible subgroup of group I.

Pediococcus damnosus strain M-1 and *P. damnosus* var. *salicinaceus* strains 8519 and 8520 were readily distinguishable from the other *pediococci* by their lower optimum growth temperature of 22°. They did not produce ammonia from arginine and *P. damnosus* strain M-I produced acetylmethylcarbinol from glucose but not from lactose.

Pediococcus halophilus strain Tc. 1 was exceptional in its requirement for 5% (w/v) sodium chloride; in most of its other reactions it resembled members of group III.

Aerococci. These organisms were fairly uniform in character. They resembled the *pediococci*, as defined above, in morphology, failure to hydrolyse gelatin, to reduce nitrate to nitrite or to utilize ammonia salts as sole source of nitrogen, and in absence of gas formation from glucose. Unlike the *pediococci*, however, the *aerococci* grew well in nutrient broth, grew vigorously at pH 9.0 and were highly sensitive to acidity (even pH 6.8 exerted an inhibitory effect). Nine of the 11 isolates were catalase-positive, one was negative and one gave a variable reaction. However, in the media used all these *aerococci* were strictly aerobic.

DISCUSSION

Relationship of pediococci to other genera

The results of the present work provide additional evidence in support of the recognition of a separate genus *Pediococcus* as suggested by Balcke (1884), Mees (1934) and more recently by Pederson (1949), Felton & Niven (1953) and Jensen & Seeley (1954). All members of this genus are easily recognizable by their morphology, mode of division and high lactic acid-producing capacity. We think, however, that the genus should include a rather wider range of organisms than suggested by Pederson *et al.* (1954) and by Jensen & Seeley (1954).

The present results show that the leucovorin (folinic acid) requirement, suggested by Felton & Niven (1953) and by Jensen & Seeley (1954) to be typical of all *pediococci*, is, in fact, restricted only to some isolates within our group I. Another property considered by some investigators to be an outstanding character of *pediococci* (Balcke, 1884; Lindner, 1887; Claussen, 1903) was the ability to multiply vigorously in beer, although more recently strains have been described which failed to multiply in this medium (Mees, 1934; Shimwell, 1949; Pederson, 1949). Pederson (1957) in *Bergey's Manual* (7th ed.) describes the genus as producing acidification and some degree of clouding in beer, and the two species listed are separated according to their optimum growth temperature and ability to grow in beer and hopped wort. *Pediococcus cerevisiae* Balcke 1884, the type species, is regarded as capable of growth in wort, hopped wort and beer, while *P. acidilactici* Lindner 1887 will grow in unhopped

wort but not in beer. Of the isolates studied in the present work, none showed growth in the particular beer used for the test. The use of the criterion 'growth in beer', without further qualifications as to the kind in which growth is tested, is however of little value, as beers may show wide variation in acidity, in hop content, in ethanol and carbon dioxide concentration, and in the degree of 'attenuation' which influences the quantity of nutrients available in the medium.

The present investigation confirms the separation of the genus *Pediococcus* from the other closely related genera. It resembles the lactic acid streptococci in requiring complex media for growth as shown by Jensen & Seeley (1954), and in being homofermentative as demonstrated by Pederson *et al.* (1954) and confirmed in this study. However, as reported previously (Günther, 1959), the pediococci are also clearly distinguishable from streptococci on the basis of morphology and mode of division. Morphology, homofermentative character and production of optically inactive lactic acid, as found in the present work and previously by Pederson *et al.* (1954) serve to differentiate the pediococci from the genus *Leuconostoc*.

The pediococci closely resemble micrococci in morphology and mode of division but may be separated from them by consideration of their biochemical characters. Although biochemical characters are often found to be variable and therefore unreliable as diagnostic criteria, all of those investigated in the present study have been shown to be stable over a period of at least 18 months. Where isolates obtained by other authors were investigated, the results were in general the same as those previously described. Therefore it seems justifiable to use such characters as differential criteria. Thus pediococci can be differentiated from micrococci on the basis of their failure to: grow on simple media; utilize ammonium salts as sole source of nitrogen; reduce nitrate to nitrite or nitrogen gas; liquefy gelatin.

The differentiation between pediococci and aerococci is less satisfactory on the basis of present results. Their morphology and mode of division is similar and neither group is capable of reducing nitrate, hydrolysing gelatin or utilizing ammonium salts as sources of nitrogen. Jensen & Seeley (1954) and Dr Ellen I. Garvie (personal communication) have suggested that the two groups may be related sufficiently to be included in the same genus. However, in contrast to the pediococci, the aerococci grew well on simpler media, grew only under aerobic conditions, were highly sensitive to acid, but grew profusely in alkaline media (pH 9.0). Such differences provide sufficient evidence for separating the two groups but further experimental data are yet required before establishing their separation at generic or specific level.

Subdivision of the pediococci

A subdivision of the pediococci into three groups, as indicated in the section on results, is suggested. Although there is some variability within each proposed group, experience in the laboratory handling of these organisms has enabled us to recognize easily the three groups by their cultural characters and we feel justified in suggesting the subdivision of the pediococci in this way.

Pederson (1949) and Jensen & Seeley (1954) recognized only one species, *Pediococcus cerevisiae* Balcke, the type species. In a later paper, Pederson *et al.* (1954) suggested two possible additional species of which the first (*a*) produced slime, and the second (*b*) possessed a higher optimum growth temperature. For organism (*a*) the name *P. viscosus* Lindner was suggested, and for organism (*b*) either *P. acidilactici*

Lindner or *P. hennebergi* Sollied. In the seventh edition of *Bergey's Manual*, Pederson (1957) lists two species: the type species *P. cerevisiae* Balcke; *P. acidilactici* Lindner characterized by an optimum growth temperature of 40° and failure to grow in beer. In the present survey no isolates were observed which produced slime (in presence of sucrose) or had a high optimum temperature.

The reactions characteristic of the group I organisms of this study are in general the same as those described for the strains of *Pediococcus cerevisiae* Balcke studied by Pederson (1949), Felton & Niven (1953), Jensen & Seeley (1954) and Dacre (1958*a*). It would appear justifiable, therefore, to apply the species name *P. cerevisiae* Balcke to this group, which includes the isolates received as *P. pentosaceus* (Mees), strain A2 (NCDO 559) of Dacre (1958*a*), and strain P-60 previously known as *Leuconostoc mesenteroides* P-60 but classified recently with the pediococci by Garvie (1959).

Pederson *et al.* (1954) compared strain *Pediococcus urinae equi* with their culture of *P. cerevisiae* and concluded that this organism should not be included in the pediococci because of lower acid-production properties. The present study confirmed the inability of *P. urinae equi* to produce a low final pH value but showed that it was indistinguishable from *P. cerevisiae* in most of its morphological and cultural characters. It can therefore only be regarded as a variant of that species. The features of the group II isolates are sufficiently distinct to warrant the recognition of a separate species. No description could be found in the literature of such a species and the name *Pediococcus parvulus* is suggested.

Group III is much less well defined. The characters of this group resemble those described by Andrews & Gilliland (1952) for a dextrin-fermenting organism which they named *Streptococcus damnosus* var. *diastaticus*. A culture of the latter organism was not available for comparative study. It is felt that insufficient evidence is at present available on which to base the establishment of this group at specific rank.

Three isolates received as *Pediococcus damnosus* strain M.1 and *P. damnosus* var. *salicinaceus* strains 8519 and 8520 failed to fit into any of the three groups described. None of them fermented salicin. Many authors have found the fermentation of pentoses and salicin to be variable and Pederson (1949) classified such strains as *P. cerevisiae*. However, the three isolates we received resembled each other and differed from *P. cerevisiae* (group I) in a number of characters (see Table 2; it may be noted that they all have a low optimum temperature). They might form the nucleus of a fourth group should other isolates with such characters be noted in future and should be regarded as members of a species *P. damnosus*.

The culture received as *Pediococcus halophilus* (Tc.1) was quite distinct in character (especially in its requirement for 5 %, w/v, NaCl) and, should additional strains be isolated, a fifth group might be recognized. Deibel & Niven (1960) described strains of pediococci isolated from meat-curing brines which were salt tolerant and produced dextrorotatory lactic acid from glucose. Deibel & Niven considered that their strains may be closely related to the marine micrococcus *Gaffkya homari* (Sniesko & Taylor, 1947) and also to *Aerococcus viridans*. They suggested that all these organisms should be placed in the genus *Pediococcus* with the species name *Pediococcus homari* nov.comb. Further investigation is needed before it can be decided whether this species and the *P. halophilus* of Mees (1934) are the same. It

would seem that a group of salt-tolerant or even halophilic pediococci might indeed constitute a fifth species.

Serological work to be published in the following paper (Günther & White, 1961) confirms a subdivision into at least three groups as here suggested.

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Serological Characters of the *Pediococci*

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SUMMARY

Rabbit antisera were prepared against 12 *pediococcus* isolates and tested by precipitin, precipitin-absorption and agglutination tests. Common precipitins for the three species *Pediococcus cerevisiae*, *P. parvulus* n.sp. and *P. damnosus* were demonstrated. An antiserum prepared against *Aerococcus viridans* (NCTC 7599) reacted with extracts of the homologous strain and 7 out of 10 heterologous *Aerococcus viridans*.

INTRODUCTION

Renewed interest in the genus *Pediococcus* has led in recent years to extensive studies of the cultural and physiological characters of its members (Pederson, 1949; Felton & Niven, 1953; Pederson, Albury & Breed, 1954; Jensen & Seeley, 1954) but, so far, no study has been reported of the serological properties of the organisms. In view of the successful application of precipitin and agglutination tests in the characterization of other lactic acid bacteria it was decided to explore the possibility of using serological methods in the classification of *pediococci*. Some preliminary results of this investigation are reported in this paper.

METHODS

The cultures and methods of cultivation are described in our previous paper (Günther & White, 1961).

Organisms. Twelve strains representative of the physiological groups described in the same previous publication were selected for antisera production.

Pediococcus cerevisiae (group I) was represented by strains 8081, F-166, 2-170, N-91, A-1, FP-1; *P. parvulus* (group II) by strains M-31, A-181, S-190, SS-69; *P. damnosus* (possible group IV) by strain M-1, and *P. halophilus* (possible group V) by strain Tc.1.

We intended to prepare an antiserum for strain L-148, which belongs to the unnamed group (group III), but this proved unsatisfactory and no further attempts have as yet been made to prepare antisera against strains belonging to this group.

One antiserum against *Aerococcus viridans* H-9 (NCTC 7599) was prepared.

Preparation of antisera. Intravenous injections of (a) whole living organisms, or (b) disintegrated suspensions, were given to rabbits which had been tested previously for the absence of naturally occurring antibodies. For method (a) 40 ml. glucose Lemco (GL) broth (Shattock & Hirsch, 1947) were inoculated with 0.5 ml. of a 24 hr. 'Oxoid' tomato juice (TJ) broth culture and incubated for 24 hr. at 30°. The culture was centrifuged, washed twice in 0.9% (w/v) sodium chloride solution

resuspended in 0.9% sodium chloride solution to give an opacity equivalent to Brown's tube No. 7 and then stored at 4° for use in one course of animal injections. In method (b) fresh suspensions of organisms were prepared for each injection. Organisms from 20 ml. of a culture in GL broth were washed and adjusted to the same optical density as in method (a). They were then disrupted for 30 min. at room temperature in a tissue disintegrator (Mickle, 1948) in a vessel containing 5 g. sterile Ballotini beads grade No. 12. After centrifugation the supernatant fluid was found to be sterile and was used for animal injection.

When the two methods of antibody production were compared it was noted that the disintegration of the organism before inoculation did not result in a speedier production of antibodies, nor was the potency of the resulting antiserum increased. Suspensions of whole living organisms were therefore used for the routine preparation of antisera; the results recorded below refer in all instances to sera prepared by this method.

The experimental animals received 1 ml. volumes of suspensions of organisms prepared as above at 3-4 days intervals. One course of injections consisted of five such inoculations. When a test bleeding at this stage indicated that the antibody produced was of insufficient potency, a second course was given. Occasionally, a third course had to be administered before satisfactory antibody was obtained. The animals were bled 3 or 4 days after the last injection. Sera were stored at 4° without preservative.

The presence of non-specific antibodies was excluded by precipitin tests with each serum against extracts of streptococci (representative of Lancefield's groups A to N) and against extracts of 7 staphylococcus and 3 leuconostoc strains.

Preparation of extracts. (a) *Crude extracts:* Lancefield's (1933) HCl extraction method was used as modified by Sharpe (1955). The cultures were incubated for 48 hr. at 30° in tomato juice broth. The reactions of at least two extracts, prepared at different times, were tested to ensure reproducibility of results. (b) *Ethanol precipitation.* The difficulty in reading the results with some of the sera and crude extracts prepared as above made it desirable to concentrate the antigenically active material in the extracts by ethanol precipitation as suggested by Shattock (1949) for group D streptococci. A slight increase in activity in the precipitate was noted with most of the extracts, but in a few this treatment resulted in the complete loss of activity in the precipitate as well as in the supernatant fluid. Ethanol precipitation was not used, therefore, for the routine preparation of extracts.

Precipitin tests. Ring tests were carried out as described by Sharpe (1955). Observations were continued for 30 min., or up to 60 min. when a loss of potency in the antiserum was noted after prolonged storage. Results obtained with antisera which had been stored for a long time were always regarded as unreliable but were useful in preliminary work.

Precipitin-absorption tests. The organisms were cultivated in tomato juice broth for 24 hr. at 30°. The technique outlined by Sharpe (1955) was followed.

Agglutination tests. Tube agglutination, employing suspensions of organisms from 16 hr. tomato juice broth cultures in 0.9% (w/v) sodium chloride solution, was used. The tubes were read after overnight incubation at 50°.

RESULTS

The selected *pediococcus* isolates differed in their capacity to induce antibody response in the rabbit and the choice of suitable isolates for antiserum production was of great importance. The difficulty experienced in obtaining active extracts with some isolates merits further investigation. In the future, modifications may

Table 1. *Precipitin tests with antisera of pediococci and Aerococcus viridans*

	Antisera												
	<i>P. cerevisiae</i>						<i>P. parvulus</i>				<i>P. dam-</i>	<i>P. halo-</i>	<i>A. viri-</i>
	F-166	2-170	8081	N-91	A-1	FP-1	M-31	A-181	S-190	SS-69	nosus	philus	dans
											M-1	Tc.1	H-9
<i>cerevisiae</i> (group I)													
F-166	++	+	+	+	+	+	+	+	+	+	±	—	—
2-170	+	++	+	±	±	+	+	+	+	±	—	—	—
8081	+	+	++	+	+	+	+	+	+	+	+	—	—
N-91	+	+	+	++	+	+	+	±	—	+	—	—	—
A-1	+	+	+	—	++	+	—	—	—	—	—	—	—
FP-1	+	+	+	—	—	++	+	—	—	+	—	—	—
Others	$+\frac{30}{31}$	$+\frac{29}{31}$	$+\frac{27}{31}$	$+\frac{24}{30}$	$+\frac{26}{30}$	$+\frac{29}{31}$	$+\frac{24}{30}$	$+\frac{14}{16}$	$+\frac{18}{30}$	$+\frac{28}{30}$	$+\frac{21}{28}$	$-\frac{28}{28}$	$-\frac{30}{30}$
<i>parvulus</i> (group II)													
M-31	+	+	+	+	.	+	++	+	—	+	+	—	—
A-181	+	+	±	—	+	±	+	++	+	+	+	—	—
S-190	+	+	+	+	+	+	+	.	++	—	—	—	—
SS-69	+	+	+	+	±	±	+	.	—	++	+	—	—
Others	$+\frac{20}{24}$	$+\frac{21}{24}$	$-\frac{17}{24}$	$-\frac{17}{24}$	$-\frac{14}{18}$	$+\frac{10}{24}$	$+\frac{22}{24}$	$-\frac{9}{13}$	$-\frac{18}{23}$	$+\frac{18}{24}$	$+\frac{18}{24}$	$-\frac{24}{24}$	$-\frac{24}{24}$
<i>damnosus</i>													
? group IV)													
M-1	+	±	±	—	—	—	+	.	—	+	++	—	—
Others	$+\frac{2}{2}$	$+\frac{2}{2}$	$+\frac{1}{2}$	$+\frac{1}{2}$.	$-\frac{2}{2}$	$+\frac{2}{2}$.	$-\frac{2}{2}$	$+\frac{1}{2}$.	$-\frac{2}{2}$	$-\frac{2}{2}$
<i>halophilus</i> Tc.1													
? group V)													
<i>viridans</i>													
H-9	—	—	—	—	—	—	—	—	—	—	—	—	++
Others	$-\frac{8}{10}$	$-\frac{10}{10}$	$-\frac{9}{10}$	$-\frac{10}{10}$	$-\frac{3}{3}$	$-\frac{9}{9}$	$-\frac{9}{9}$	$-\frac{1}{1}$	$-\frac{8}{10}$	$-\frac{9}{10}$.	$-\frac{10}{10}$	$+\frac{7}{10}$
<i>eucnostoc</i>													
	$-\frac{3}{3}$	$-\frac{2}{3}$	$-\frac{3}{3}$.	$-\frac{3}{3}$	$-\frac{3}{3}$.	$-\frac{3}{3}$
<i>treptococci</i>													
	$-\frac{24}{24}$	$-\frac{24}{24}$	$-\frac{24}{24}$	$-\frac{12}{24}$	$-\frac{12}{24}$	$-\frac{24}{24}$	$-\frac{12}{24}$	$-\frac{24}{24}$	$-\frac{12}{24}$	$-\frac{12}{24}$	$-\frac{12}{24}$	$-\frac{12}{24}$	$-\frac{24}{24}$
<i>taphylococci</i>													
<i>Staph. aureus</i> 7447	—	—	—	—	.	—	—	.	—	—	—	+	+
<i>Staph. albus</i> 7292	—	—	—	—	.	—	—	.	—	—	—	+	+
Others	$-\frac{5}{5}$	$-\frac{5}{5}$	$-\frac{5}{5}$	$-\frac{5}{5}$.	$-\frac{5}{5}$	$-\frac{5}{5}$.	$-\frac{5}{5}$	$-\frac{5}{5}$	$-\frac{4}{5}$	$-\frac{3}{5}$	$-\frac{4}{5}$

++ = strong precipitate, + = precipitate, ± = weak reaction, — = no reaction, . = not tested.

The figures are $\frac{\text{no. of strains giving reaction}}{\text{no. of strains tested}}$.

have to be made in the method of preparation in order to obtain satisfactory extracts of consistently high activity.

Precipitin tests. These results are summarized in Table 1. Positive reactions were obtained with extracts of the homologous and with the majority of heterologous strains of the same species. Antisera against *Pediococcus cerevisiae* strains F-166, 2-170, and FP-1 also precipitated consistently with extracts of *P. parvulus*. Antisera against *P. cerevisiae* strains 8081, N-91, and A-1 were less consistent in their reactions with extracts of *P. parvulus*, but they too gave positive reactions with a large number of extracts of that species.

Antisera against *Pediococcus parvulus* and *P. damnosus* reacted with the homologous and with the majority of the heterologous extracts of the same species as well as with extracts of *P. cerevisiae*. Cross-reactions occurred also between *P. parvulus* and *P. damnosus*.

Reactions could not be observed between sera against *Pediococcus cerevisiae*, *P. parvulus* or *P. damnosus* on the one hand, and between 'group III' isolates or *P. halophilus* on the other. The serum against *P. halophilus* was not precipitated by extracts of any of the other pediococci.

Table 2. *Absorption of antisera of Pediococcus cerevisiae strains F-166 and 2-170 with cells of homologous and heterologous P. cerevisiae strains*

Antigen (extracts)	Antiserum									
	F-166					2-170				
	Unab- sorbed	Absorbed with strains			Unab- sorbed	Absorbed with strains				A-1
		F-166	D-32	PUE		2-170	F-166	8081	FP-1	
<i>P. cerevisiae</i> (group I)										
F-166	++	—	—	+	+	—	—	—	—	—
2-170	+	—	.	—	++	—	—	.	.	.
PUE	+	—	.	—	+
Others	+	—	—	—	+	—	—	—	—	—
<i>P. parvulus</i> (group II)	+	—	—	—	+	.	.	—	—	—

Symbols as Table 1.

Six of the pediococcus antisera gave positive reactions with one or the other aerococcus extract, but in these instances ring formation was markedly delayed and considerably less intense than that observed with the pediococci.

The *Aerococcus viridans* H-9 serum reacted with extracts of the homologous and with 7 of the 10 other strains of *A. viridans* tested. A weak cross-reaction was obtained also with extracts of *Pediococcus halophilus* and with strain L-171 of the unclassified group III.

Cross-reactions were not found between the pediococcus antisera used and the extracts of streptococci, staphylococci and leuconostoc strains, with the exception of one reaction between a strain of *Staphylococcus albus* and *Pediococcus halophilus* antiserum.

Precipitin-absorption tests. Four antisera were selected for absorption tests. The results are shown in Tables 2, 3, 4. These results indicate that certain antisera contained more than one antibody, one of which was absorbed by heterologous

Table 3. Absorption of antiserum *Pediococcus cerevisiae* strain F-166 with cells of *P. parvulus* and strain L-148 (group III)

Antigen	Antiserum F-166 absorbed with strains <i>P. parvulus</i>					
	Un-absorbed	A-181	S-190	SS-69	S-182	L-148
<i>P. cerevisiae</i> (group I)						
F-166	++	+	—	+	—	+
2-170	+	—	.	—	.	+
PUE	+	.	—	.	.	.
Others	+	—	—	—	—	.
<i>P. parvulus</i> (group II)	+	—	—	—	—	+

Symbols as Table 1.

Table 4. Absorption of antisera *Pediococcus parvulus* strains S-190 and SS-69 with cells of *P. cerevisiae* and *P. parvulus*

Antigen	Antiserum							
	S-190				SS-69			
	Unabsorbed	Absorbed with <i>P. parvulus</i>		<i>P. cerevisiae</i> F-166	Unabsorbed	Absorbed with <i>P. parvulus</i>		<i>P. cerevisiae</i>
		S-190	SS-69			SS-69	F-166	PUE
<i>P. parvulus</i> (group II)								
S-190	+	—	+	+	+	—	—	—
SS-69	+	.	.	.	+	—	+	+
Others	+	—	—	—	+	—	—	—
<i>P. cerevisiae</i> (group I)	+	—	—	—	+	—	—	—

Symbols as Table 1.

Table 5. Agglutination tests with *pediococcus* and *aerococcus* antisera

Antigen	Antisera							
	<i>P. cerevisiae</i>					<i>P. parvulus</i>		<i>A. viridans</i> H-9
	F-166	2-170	8081	N-91	FP-1	M-31	SS-69	
<i>P. cerevisiae</i> (group I)								
F-166	1280+	—	—	—	—	—	—	—
2-170	80	1280+	—	40	80	—	—	—
8081	20	40	1280+	40	40	40	40	20
N-91	—	—	—	640	—	—	—	—
FP-1	40	80	—	40	1280+	—	160	—
<i>P. parvulus</i> (group II)								
M-31	—	—	—	—	—	1280+	—	—
SS-69	320	40	40	40	160	80	1280	40
<i>A. viridans</i> H-9	—	—	—	—	—	—	320	1280+

Figures are the reciprocals of the highest titres read. — = > 20.

strains and the other absorbed only by the homologous strain. Antibodies in *Pediococcus cerevisiae* antiserum were absorbed by *P. parvulus* strains and vice versa, indicating that these two species share at least one common antigen. Absorption with strain L-148 organisms, with strains of *Aerococcus viridans*, streptococci and staphylococci did not result in loss of antibodies from the antisera of *P. cerevisiae* or *P. parvulus*.

Agglutination tests. The results are shown in Table 5. With homologous strains titres of more than 1/1280 were obtained generally but with heterologous strains, when any reaction at all was obtained, the titres seldom exceeded 1/80. A number of cross-reactions occurred between antisera of *Pediococcus cerevisiae* and *P. parvulus*. In one instance cross-reactions occurred between *P. parvulus* strain SS-69 and *Aerococcus viridans* strain H-9.

DISCUSSION

The present investigation indicates that serological techniques may be applied successfully to the separation and classification of the pediococci. Antisera prepared against pediococci did not react with extracts of organisms from the closely related genera *Streptococcus* and *Leuconostoc* and generally not with extracts from aerococci. It becomes evident that pediococci possess precipitins which are common to members of more than one species. Such antigens were demonstrated in *Pediococcus cerevisiae*, *P. parvulus* and *P. damnosus*, but not in *P. halophilus* and in members of the as yet unclassified subgroup (III). As these antigens were shared by more than one species they may be referred to as 'group' antigens by analogy to the group antigens of streptococci.

Some pediococcus strains showed an additional antigen which appeared to be more strain specific and may therefore represent a 'type' antigen. The presence of more than one antigen was also demonstrated in agglutination tests. These tests revealed relationships apparently much narrower than were demonstrated in precipitin tests. It is probable, then, that pediococci possess a 'group' antigen demonstrable by precipitin tests and a 'type' antigen which can be shown by precipitin and agglutination tests, as with streptococci and lactobacilli. No study has as yet been made to determine whether the 'type' antigens demonstrable in the two tests were identical. The possibility that the organisms possess more than one 'type' antigen cannot be excluded.

Some indication that the 'group' antigen may be protein in nature was obtained from ethanol precipitation of some of the extracts since the antigenically active material had been found in the precipitate. However, the antigen might be carbohydrate carried down with the protein.

The serological position of *Pediococcus halophilus* and of the unnamed group III could not be established satisfactorily. Absorption of *P. cerevisiae* antiserum with one strain of group III did not remove the 'group' antigen from the serum. This indicates that members of group III are not related antigenically to *P. cerevisiae* (group I) and *P. parvulus* (group II). They may possibly represent a separate serological group.

The reaction of strain PUE is of interest. Pederson *et al.* (1954) excluded it from the pediococci but the strain was classified by us (Günther & White, 1961) as *Pediococcus cerevisiae*. This organism gave strong precipitin reactions with antisera

of *P. cerevisiae*, *P. parvulus* and *P. damnosus*. When PUE was used for absorption of *P. cerevisiae* antiserum the 'group' antigen was absorbed. This is further evidence that the correct classification of this strain is with *P. cerevisiae*.

The serological work also confirms the classification of strain A-2 isolated by Dacre and classified by him tentatively as *P. cerevisiae* (Dacre, 1958) and of the old '*Leuconostoc mesenteroides* P-60' reclassified by Garvie (1959) as *P. cerevisiae*.

The cross-reactions between the aerococci and pediococci might have been due to non-specific antibodies, but more experimental evidence is required. The work is as yet incomplete but it is being continued and we hope to clarify at least some of the issues raised in this and the preceding paper.

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The Production of Sporidesmin and Sporidesmolides by *Pithomyces chartarum*

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SUMMARY

Methods for the assay of sporidesmin and the sporidesmolides were worked out and used in the study of the production of these metabolites by *Pithomyces chartarum* (Commonwealth Mycological Institute, Kew, England, Herbarium no. 74473) growing on an enriched potato carrot medium. High yields of sporidesmin and the sporidesmolides were associated with high utilization of medium constituents. No increase in the quantity of sporidesmin isolated from unit volume of medium was observed after the third day of incubation, despite further rapid growth of the fungus; by contrast good correlation was observed between the weight of sporidesmolides produced and fungal growth. These results were successfully applied to the production of experimental quantities of these materials.

INTRODUCTION

Recently the suggestion was made (Percival & Thornton, 1958; Thornton & Percival, 1959) that the production of toxic material by a micro-organism growing on pasture flora was an important part of a disease of sheep and cattle which occurs in North Island, New Zealand, and is known as facial eczema. The disease occurs under climatological conditions which are conducive to fungal growth. Sheltered warm low-lying pastures have frequently had a history of facial eczema outbreaks. In a survey Thornton & Ross (1959) showed that certain micro-organisms were predominant on pastures where the disease commonly occurred. One of these micro-organisms *Pithomyces chartarum* (Berk. & Curt.) Ellis (1960) (syn. *Sporidesmium bakeri* Syd.; Commonwealth Mycological Institute Herbarium no. 74473) after surface-culture on potato carrot medium for 7 days was fed to sheep. The livers of these animals showed pathological changes similar to those observed in clinical cases of facial eczema (Percival, 1959). Chemical examination (Russell, 1960) of such cultures showed that depsipeptides (Shemyakin, 1960) probably identical with those isolated from cut grass from pastures where facial eczema had occurred (White, 1958) were present. Synge & White (1959) isolated from ethereal extracts of cultures of this fungus a crystalline material which they named sporidesmin. This material, administered orally to guinea-pigs, produced pathological changes in the livers which resembled those observed when similar experimental animals were fed

samples of dried grass from pastures known to have been associated with the disease. The potential importance of factors of this kind seemed to warrant an attempt to find what conditions of laboratory cultivation were conducive to the growth of the fungus and whether these were related to the formation of sporidesmin. As the sporidesmolides belong to a group of natural products which commonly possess antibiotic activity (Shemyakin, 1960) it was also of interest to find whether their appearance in the cultures was related to the production of sporidesmin and to the growth of the mould. Such experiments might find application in pasture management, in comparisons of the ability of different strains of the fungus to produce toxic metabolites and in the production of adequate quantities of sporidesmin and the sporidesmolides for biochemical and pathological comparisons of the disease as observed in the field with that produced experimentally.

METHODS

Organisms. There is at present some controversy about the nomenclature of the organism used in this work (see Hughes, 1958; Ellis, 1960). With one exception the strain used in the present experiments was disposed by the Commonwealth Mycological Institute (Kew, England) as *Sporidesmium bakeri* Syd. (Hughes, 1953), C.M.I. Herbarium no. 74473. The name *Pithomyces chartarum* is used in this paper on the basis of Ellis's (1960) work. Miss J. M. Dingley provided us with a strain of this organism that she had isolated at Mount Albert, Auckland, New Zealand. This strain (Ellis, 1960) was used in the experiments recorded in Table 3.

Inocula. Sterile 0.05% (w/v) Lissapol N samples (10 ml.) were transferred aseptically to a 10- to 21-day culture of the fungus on potato carrot agar prepared from the medium described below diluted to half strength with tap water with omission of the additional glucose. The liquid was washed over the slope until this was thoroughly wetted. The suspension was decanted into a sterile container attached to an automatic pipette and sterile water added to make the spore concentration 5×10^6 spores/ml. For larger quantities of inocula sterile Lissapol N solution (50 ml., 0.05% w/v; I.C.I. Ltd.) was added to a 21-day culture of the organism on rye (*Secale cereale*) and the spore suspension prepared as before. The suspension (0.1 ml.) was inoculated to the medium (100 ml.) and to each milk bottle of rye grain or bran (see below).

Surface cultures. Potatoes (30 kg.) and carrots (30 kg.) were scrubbed and cut mechanically into 3 cm. cubes. The mixed vegetables were covered with 60–70 l. tap water in a stainless steel vessel equipped with a stainless steel stirrer (120 rev./min.) and three 2 kW. immersion heaters. The stirred mixture was boiled for 30 min. after which the semi-solid material was allowed to settle and the supernatant liquor decanted. The liquor was made up to the required volume (about 100 l.) and sugar and nitrogen determinations carried out. The sugar concentration was then adjusted to 1% (w/v) by adding glucose and the nitrogen content was made to 0.05% (w/v) by adding ammonium sulphate. The medium was adjusted to pH 5.0 by adding dilute sulphuric acid. After filling the culture vessels the medium was heated at about 120° for 20 min. All surface cultures were incubated at $24^\circ \pm 0.5^\circ$ for 7 days.

Cultures on rye grain and on bran. In each of 200 pint milk bottles were put rye grain (75 g.) and tap water (45 ml.) (or 50 g. bran and 20 ml. water) and the bottles

allowed to stand overnight. The bottles were then plugged with non-absorbent cotton wool, the plugs being protected with aluminium foil. The bottles were autoclaved at about 120° for 2 hr. After standing in the incubation room for 48 hr. three samples selected at random were removed aseptically, transferred to potato + carrot agar slopes and incubated at 24° for 72 hr. These slopes were examined from time to time. Meanwhile, when no obvious contamination was present, the bottles were inoculated and incubated at $24^{\circ} \pm 0.5^{\circ}$ for 28 days.

Extraction. After incubation for 28 days 80 % (v/v) methanol in water (250 ml.) were added to each bottle, the fungus and grain broken up with a stout glass rod and the mixture poured into a stainless steel vessel. The mixture was stirred for 20 hr. at room temperature and then filtered through a large nutsche dressed with Terylene cloth (coarse-grade sail cloth available in New Zealand was used, precise specifications not known). The residue that remained on the filter was replaced in the stainless steel vessel, 80 % (v/v) methanol in water (50 l.) added and the mixture stirred for 3 days at room temperature. The liquid that had passed through the Terylene was filtered through Whatman no. 31 filter paper and the perfectly clear filtrate was evaporated down to 3 l. in a cyclone evaporator. The concentration process was operated at 2–5 mm. Hg pressure, the heating heat-exchanger was maintained at 80° and the condensing heat-exchanger at -40° . The rate of flow into the heating heat-exchanger was such that the emergent fluid from this part of the apparatus did not exceed 35° . The concentrate was then extracted with diethyl ether (peroxide free, 1 l.) continuously in a Hilditch-type fat extractor for 18 hr. The concentrate may also be extracted by hand in a separating funnel in an efficient fume cupboard but even so the process is hazardous; severe dermatitis was experienced by workers using this technique in the early stages of this work. The ethereal extract was evaporated to dryness *in vacuo*. The second methanolic extract was processed similarly. Gas chromatographic analysis of the methanolic extracting solvent indicated this to be 60–70 % (v/v) methanol in water. The average yield at this stage for 20 batches was 1.2 g. sporidesmin as a 10–20 % (w/v) concentrate. Concentrates of about 20 % (w/v) were obtained when *isopropyl* ether was used as the second extractant. The lowest yield was 0.6 g. and the highest 1.9 g. Alumina chromatography of this concentrate, partition of the diethyl ether eluate between 80 % (v/v) methanol in water and light petroleum (b.p. $60-80^{\circ}$) and finally partition chromatography on Hyflo-supercel with the system carbon disulphide + methanol + water (5, 4, 1, by vol.) as described by Syngé & White (1959), provided crystalline material in 80–90 % overall recovery. This material was used in animal experiments to be described elsewhere.

The rye grain and fungus from the second methanolic extraction was stirred and boiled with methanol (20 l.) for 3 hr. and the mixture filtered hot. The hot filtrate was concentrated to 4 l. in a cyclone evaporator. The concentration process was operated at 20 mm. Hg, the heating heat-exchanger was maintained at 110° and the condensing heat-exchanger at -40° . The concentrate was kept in the cold room at 2° for 24 hr. and the solid that had separated was collected and dried *in vacuo*. The average yield for ten batches was 27 g. containing 50–70 % sporidesmolides. Further purification was achieved as described below.

Analytical methods. As only about 50 % of the sporidesmin produced by the growth of *Pithomyces chartarum* was found in culture filtrates all analyses were

done on extracts of culture medium and mycelium. Extracts were prepared as follows. The fungus separated from the culture medium was macerated with Whatman no. 31 filter paper (one 12.5 cm. filter paper/100 g. wet wt. fungus) in a top-drive macerator; the resulting mixture was filtered and the residue sucked dry. The filtrate was added to the culture medium and the residue macerated under diethyl ether (400 ml./500 g. wet wt. residue). This process was repeated three times and the combined ethereal solutions, residual fungus and culture medium were continuously extracted in a Hilditch-type fat extractor for 20 hr. At least 1 l. of ethereal extract was kept in the boiling flask which was heated in a water bath at 80°; when quantities of culture medium between 5 and 20 l. were extracted 2 l. of ethereal extract were kept in the boiling flask. Further extraction produced only traces of sporidesmin. The ethereal extract was evaporated almost to dryness at 0° and the residue cooled to -80°. The ice and sporidesmolides (about 50 mg. of the latter/l. were obtained by this procedure) were collected, washed with diethyl ether and the filtrate and washings evaporated to dryness in a tared vessel *in vacuo*. The resulting brown gum (1 g./10 l.) was assayed by at least two of the following procedures.

Tissue culture toxicity tests. Human malignant epithelial cells (strain HeLa, Gey) were obtained from Mr A. M. Murphy (Auckland General Hospital) in May 1959 and transformed pig kidney cells from Glaxo Laboratories Ltd. in October 1959. The medium described by Murphy & Worker (1960) was modified by increasing the calf serum content to 10% (v/v) and by the addition of penicillin (6 µg./ml. = 100 i.u./ml.) and streptomycin (100 µg./ml.). Phosphate buffered saline (PBS) was made up with sodium chloride (A.R., 1.15 g.), potassium chloride (A.R., 0.2 g.), disodium hydrogen phosphate (A.R., 1.15 g.), potassium dihydrogen phosphate (0.2 g.) and water to 1 l. A 0.1% (w/v) 'Bactotrypsin 1:250' solution in PBS was used for trypsinization. Water for the medium, PBS and trypsin was twice glass-distilled. Stock cultures were maintained in 150 ml. square Pyrex screw-cap bottles and test cultures were grown in 125 × 16 mm. Pyrex screw-cap test-tubes. After use these vessels were soaked overnight in 5% (w/v) potassium hydroxide solution in ethanol; they were then washed in tap water, with dilute hydrochloric acid (about 2%, w/v, HCl in water) and finally with distilled water.

The medium was decanted from a 7- to 10-day bottle culture and the cells treated with the trypsin solution (8 ml.). After incubation for 10–15 min. the contents of the bottles were gently mixed and the vessel clamped so that the cells settled in one corner of the base. The supernatant fluid was sucked off and fresh medium (5 ml.) added. A uniform suspension was made by making the cells enter and leave a pipette 5–10 times (the efficiency of a pipette in this operation seems to depend on the characteristics of its tip); then a further 10 ml. portion of medium was added. A sample (0.5 ml.) of the suspension was centrifuged at low speed for a few minutes and the cells washed twice with PBS (2 × 1 ml.). Protein was estimated by the Folin colorimetric method (see below). Medium was then added to the suspension to give a final cell-protein content of 50 µg./ml. and 1 ml. amounts were dispensed into the test-tubes, with an automatic pipette, with magnetic stirring of the suspension. In the preparation of small batches the suspension was dispensed by a 5 ml. graduated pipette. The tubes were placed in trays and the inclination adjusted until the medium reached 5–6 cm. from the bottom of the tube. The trays were rocked

gently to obtain a uniform suspension and were incubated at 36° for 24 hr. at which time extracts were added in amounts up to 0.1 ml.

Extracts were dissolved in ethanol at 20 mg./ml., and these solutions were serially diluted with PBS. Doses of 4, 2, 1, 0.5, 0.25, 0.12 and 0.06 μ g. were used for extracts from surface cultures and a tenth of these amounts for extracts from rye grain and bran cultures. Sporidesmin was assayed over the range 24, 12, 6, 3, 1.5 and 0.75 μ g./ml. from dilutions of a 50 μ g./ml. ethanolic solution.

The effects of the test solutions were usually determined by comparing the morphology of the cells in the test cultures with those in the controls after 1, 2, 3 and 4 days. The sporidesmin present was assessed on the basis of the least dose that gave a marked effect (least toxic dose). There was usually a clear difference between the effect of this dose and that of half the dose; on the other hand, the effect of twice and four times the dose appeared to be similar.

The estimation of protein in the cultures was based on the method of Oyama & Eagle (1956) and took account of the modified procedure of Miller (1959). It was found that the more concentrated sodium hydroxide solution of the modified method facilitated dissolution of the cells.

Corneal opacity test. About 10 mg. of an extract containing about 1% (w/v) sporidesmin (e.g. from a surface culture) was dispersed in 0.5 ml. of 1% (v/v) 'Tween 80' solution (L. Light and Co., Colnbrook, England) in a Griffith's tube; two four-fold serial dilutions were made and 0.05 ml. volumes of these solutions applied to the eyeballs of small New Zealand White rabbits (weighing 800–1600 g.) whilst the eyelids were held open and elevated from the eyeball. The instilled material was made to flow over the eyeball and the conjunctival membranes by gentle massage, using the elevated eyelids to disperse the material. On the following day similar amounts of the same dilutions were instilled in like manner. The eye lesions at these concentrations were compared with the lesions observed after instillation of 20, 5 and 1.25 μ g. crystalline sporidesmin applied in 1% (v/v) 'Tween 80' solution in the same way. No reaction was noticed at the greatest dilution; at the smallest dilution congestion of the scleral vessels, with oedema and inflammation of the conjunctival membranes, was observed after 2–3 days. Four to five days after instillation this concentration produced marked corneal opacity which lasted for several weeks. A similar corneal opacity was observed at the intermediate dilution 5–7 days after instillation. Controls were not normally used because no lesions were observed after instillation of 0.1 ml. 5% (w/v) 'Tween 80' solution.

Iodometric estimations. The method was essentially the same as that used for penicillin (Clarke, Johnson & Robinson, 1949). Fifty mg. extract were accurately weighed, dissolved in ethanol and the solution made up to 20 ml. with ethanol. A 5 ml. volume was treated with 5 ml. 20% (w/v) sodium hydroxide solution and the mixture allowed to stand for 15 min. at room temperature. The resulting mixture was cooled in ice-water, acidified with 30% (v/v) acetic acid in water, 10 ml. 0.01 N-iodine added and the excess iodine titrated with standard sodium thiosulphate. Starch must be used for this end-point; otherwise it is obscure because of a yellow pigment which is present in the extract. A further 5 ml. volume of the extract solution was acidified with 30% (v/v) acetic acid in water, treated with 10 ml. 0.01 N-iodine and the iodine titrated with standard sodium thiosulphate immediately. Under these conditions 1 ml. 0.01 N-iodine was equivalent to 0.7 mg.

sporidesmin and the percentage sporidesmin in the sample was calculated from the expression $(x-y)280/z\%$, where x is the titre of the blank, y the titre of the alkali-treated sample and z the weight of the original sample. Sporidesmin frequently crystallizes with one molecule of solvent (Synge & White, 1959). The conversion factor given here for the iodine titration is based on the non-solvated molecule of molecular weight 475.5.

Sporidesmolides. The solvents used were of reagent grade. Activated charcoal (Darco, Grade G. 60) was obtained from the Darco Corporation, New York, U.S.A. The total sporidesmolide fraction, prepared as described by Russell & Brown (1960), was recrystallized from 70% (v/v) acetic acid in water (m.p. 256–259°; corr.; $[\alpha]_D^{20} = -205^\circ$, $c = 1\%$, chloroform). Diethyl ether for this determination was shaken with excess of the total sporidesmolide fraction for 24 hr. and filtered before use. When sufficient mycelium was available 10 g. were used; when less was available, the volumes of the reagents, etc., were all decreased in proportion. The final volume of chloroform filtrate and washings was also adjusted as follows: for 8–10 g. mycelium, 25 ml.; 6–8 g., 20 ml.; 4–6 g., 15 ml.; less than 4 g., 10 ml.

The dried mycelial felt was weighed and extracted with methanol in a Soxhlet apparatus for 16 hr. The methanol was removed on a rotatory evaporator at 50° and the residue transferred to a separating funnel in solution in a mixture of methanol (70 ml.), chloroform (100 ml.) and water (30 ml.). The mixture was well shaken, the phases allowed to separate, the lower layer collected and the solvents removed on a rotatory evaporator as before. The residue was dissolved in a mixture of chloroform (50 ml.) and ethanol (50 ml.); then benzene (50 ml.) was added and the solvents once more evaporated. The cooled residue was treated with sporidesmolide-saturated ether (80 ml.), mixed by gentle swirling and allowed to stand at room temperature overnight. The precipitate was collected quantitatively, washed with ether saturated with sporidesmolides and treated with chloroform (about 5 ml.) at 50°. Solution of the sporidesmolides was completed by gentle agitation for 1 hr. at room temperature. Activated carbon (0.1 g.) was formed into a pad on the surface of a sintered glass funnel and the chloroform solution (containing suspended particles) of the sporidesmolides was passed through under positive pressure. The vessel that contained the unfiltered chloroform solution and the filter were washed successively with further small quantities of chloroform until the volume of the combined filtrates was 25 ml. The solution was mixed and its optical rotation determined. The weight (mg.) of total sporidesmolides (W) was calculated from the expression $W = 4.9 \alpha V/l$, where α is the observed rotation, V the volume of the solution (ml.) and l the length (dm.) of the solution.

Spore counts were made as follows. The fungus filtered off was washed on the filter with distilled water and dried to constant weight in a vacuum oven at room temperature and 0.1 mm. Hg pressure over solid sodium hydroxide. This material was also used for the determination of the amount of sporidesmolides. A sample (about 50 mg.) was weighed and ground with 0.05% (v/v) Lissapol N solution (5 ml.) until thoroughly dispersed; a duplicate sample was similarly treated. Several spore counts were done on each suspension by using a haemocytometer. The results given are the average of these counts.

Ash and dry weights. Media and filtered culture fluids were lyophilized in tared flasks to determine dry weights. The resulting solid was dissolved in the minimum

quantity of distilled water and transferred to tared silica crucibles. Solutions were evaporated to dryness in a desiccator and ashed to constant weight in a muffle furnace at 1000°.

Nitrogen was determined by the Kjeldahl method. The results (Table 4) refer to the total-N in the sample since the digests were not de-proteinized.

Sugars were determined by the method of Hanes (1929).

RESULTS

Estimations of sporidesmin and sporidesmolides were based on extracted material; thus some information was required about losses during this procedure. The sporidesmolides, because of their insolubility in water, were almost wholly present in fungal tissue. They were high melting point, cyclic depsipeptides, stable under the extraction conditions used and thus unlikely to undergo serious losses during the extraction process. Sporidesmin is unstable in alkaline solution, and parallel experiments showed decreased yields when solutions were held above 40°. Sporidesmin (5%, w/v, extract from a previous batch added in ethanolic solution to an equivalent volume of a bulked surface culture) added to a culture before the extraction process was completely recovered when submitted to the isolation procedure; further extraction of the raffinate gave only traces of sporidesmin. When a rye grain culture had been extracted five times with 80% (v/v) methanol in water some sporidesmin remained in the residue as it could be isolated by a sixth extraction. A more rapid extraction was achieved by using 80% (v/v) ethanol in water but the extract was considerably cruder. Extraction was similarly facilitated in the case of bran cultures but it was not complete and the crude isolate was only 7% sporidesmin.

Initially dilutions of fungal extracts were added to completed monolayers of HeLa cells for toxicity tests, but it was found difficult to assess a least toxic dose. Cytopathological effects at higher dilutions were mainly observed in the peripheral cells. The technique was therefore altered so that the cells were in groups when the doses were added. Reproducible results were then obtained. Toxic effects down to about the same concentration of pure sporidesmin were found by visually observed changes in cell morphology and by the more objective criterion of protein formation in cultures (Oyama & Eagle, 1956). Figure 1 shows the amounts of protein formed in cultures in the presence of different amounts of sporidesmin. The dose of sporidesmin was added in 1 ml. medium to 24 hr. cultures, and the protein estimated 5 days later. Results are expressed in terms of the amount of cell protein formed, that in untreated cultures being taken as 100. A sharp increase in protein formation occurred when doses were less than 3 m μ g. A least toxic dose of 3 m μ g. was repeatedly observed when using the visual method of assessment; this value has been used in computations of the amounts of sporidesmin in extracts of fungal material. Six fungal extracts were tested using both HeLa and transformed pig kidney cell cultures. The least toxic dose was found to be the same in four cases; in two cases the pig kidney cells dose required was twice the HeLa cell dose in order to produce a marked effect. The general appearance of the pig kidney cells at the least toxic dose concentration was characterized by shrinkage to spindle-shaped cells. HeLa cells by contrast, shrank into irregular shapes. Untreated cells of both lines were of like appearance.

Excellent agreement was usually observed (Table 1) when parallel estimations were made with the iodometric procedure and the tissue culture toxicity test. Exceptions to this were interesting and point a moral, for in these cases the biological method indicated that no, or very little, sporidesmin was present. The iodometric procedure must therefore be treated with reserve.

Sporidesmolides were determined by a quantitative adaptation of the normal isolation procedure. The recovery of sporidesmolides was determined by adding known weights of the total sporidesmolide fraction to measured quantities of a

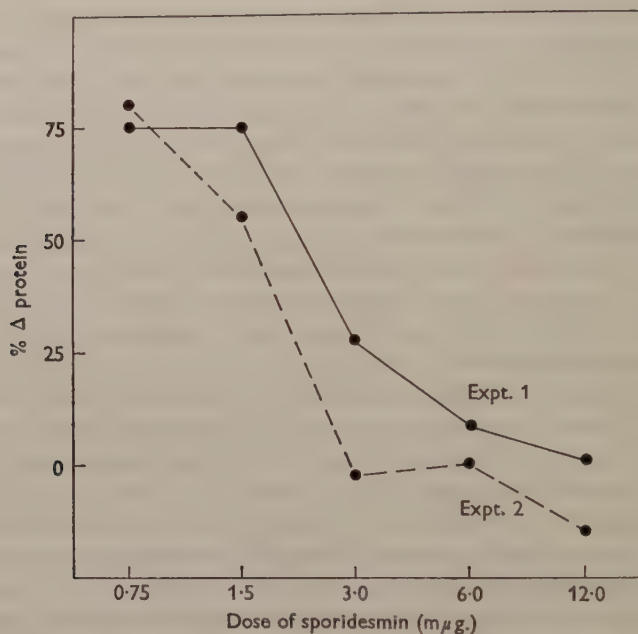


Fig. 1. Response of HeLa cells to sporidesmin doses, by colorimetric measurements of cell protein formed. Doses were added in 1 ml. amounts of medium to 24 hr. cultures which contained initially 50 μ g. cell protein/ml. medium. Measurements of protein formed were made on the sixth day. Control (no dose) cultures contained 178 μ g. (Expt. 1) and 68 μ g. (Expt. 2) cell protein. % Δ protein = protein formed as a percentage of that of controls.

methanol extract of mycelium. Quantitative recoveries of the added material were obtained. The assay provided a solution containing only sporidesmolides which current work has shown to consist essentially of two depsipeptides, closely related (Russell, 1960). The solutions were determined polarimetrically using the value obtained for the total sporidesmolide fraction of $[\alpha]_D^{20} = -205^\circ$. The accuracy of the method depended on the constancy of composition of the mixture of sporidesmolides, thus confirmatory data were obtained by determining the weight of sporidesmolides in the solutions. Significant differences were not observed between the gravimetric and polarimetric methods.

The results shown in Table 1 were selected from about 120 batches of surface cultures because the data in these batches were fairly complete and the vessel to vessel variations were small. The sporidesmin results are based on extracts from

whole batches (usually 50–100 l.). Analyses of medium dry weight were made and also nitrogen, sugar and pH determinations both on bulked filtered medium at the end of the incubation and on averages of 5–10 sample vessels selected at random. The two series of results agreed well. The mycelium dry weights given were obtained by taking the arithmetical average of the results from 5–10 sample vessels selected at random. In Table 1 the results are expressed as differences between the values obtained before and after incubation; in this way better correlation between the analyses of medium constituents and the amounts of sporidesmin isolated were obtained. In most cases, however, the figure referring to the change in medium dry weight was proportional to the concentration of the starting medium.

Table 1. *Utilization of medium constituents and sporidesmin production by Pithomyces chartarum*

Batch no.	Filtered broth Δ dry wt. (mg./ml.)	Δ N (mg./ml.)	Δ Sugar (mg./ml.)	Mycelium dry wt. (mg./ml.)	Sporidesmin		
					HeLa	R.E. (mg./l.)	I ₂
73A	—	0.12	4.7	4.0	0.3	—	0.3
63B	10.2	0.15	5.7	3.2	0.4	0.4	0.4
62	18.2	0.20	5.8	—	0.4	0.5	—
44	10.2	0.28	5.0	4.3	0.5	0.4	—
50	21.7	0.32	7.9	6.5	0.5	0.5	—
63A	18.5	0.34	6.7	5.9	0.7	—	—
111	15.7	0.34	7.0	6.1	0.7	—	0.8
68	19.1	0.55	11.8	7.9	1.0	—	1.1

The terms: 'Δ' refers to difference between analyses on starting media before inoculation and those on the final filtered broth; 'N' to nitrogen analyses; 'Mycelium dry wt.' to the total weight of fungal material; 'HeLa' to results obtained by the tissue culture method; 'R.E.' to the corneal opacity test; 'I₂' to results arising from iodometric estimations.

Table 2. *Utilization of sugars by Pithomyces chartarum*

Sugar added	Filtered broth Δ dry wt. (mg./ml.)	Mycelium dry wt. (mg./ml.)	Δ Sugar (mg./ml.)	Final [sugar]* (mg./ml.)	Δ N (mg./ml.)
None	7.3	3.1	2.14	1.10	0.22
Glucose	9.0	4.6	5.60	1.60	0.22
Lactose	8.2	4.2	4.99	3.25	0.19
Galactose	7.3	3.2	2.10	6.10	0.20
Maltose	9.1	4.5	5.62	1.75	0.21

* The square brackets in column 5 indicate concentration; other symbols as in Table 1.

In Table 2 the results of adding 0.5% (w/v) of various sugars to the culture before inoculation are summarized. These results are based on repeated experiments which compared groups of three sugars with a control. The addition of the different sugars did not increase the yield of sporidesmin though all sugars except galactose increased the amount of fungal growth during a 7-day period of incubation. Only about half the added lactose was utilized; the effect of adding this sugar on the growth of the fungus was about half that of adding glucose. Since the addition of maltose had a similar effect to the addition of glucose the partial utilization of lactose may be explained by the apparent inability of the organism to use galactose.

The addition of ammonium sulphate (Table 3) increased growth in a 7-day fermentation but not the yield of sporidesmin. The analysis of filtrates of spent broth indicated that most of the added nitrogen remained in solution, but it is not known whether this was present as unchanged ammonium salts. It seems unlikely that the increase in growth was due to the sulphur added as other ammonium salts behaved similarly.

Table 3. *Effect of adding an inorganic and an organic nitrogen source to Pithomyces chartarum strain 73a fermentations on potato + carrot media*

Adjuvant	Initial N (mg./ml.)	Final N (mg./ml.)	Mycelium dry wt. (mg./ml.)	Sporidesmin	
				HeLa (mg./l.)	I ₂
None	0.36	0.12	4.2	0.5	—
Ammonium sulphate	0.47	0.19	4.5	0.5	—
Ammonium sulphate	0.85	0.50	6.0	0.6	0.7
None	0.32	0.09	4.3	2.0	2.3
Glutamine	0.57	0.24	5.0	—	—
Glutamine	0.82	0.42	5.7	—	2.8
Glutamine	1.32	0.93	5.9	3.8	4.1

'Initial N' and 'Final N' refer to analytical values for nitrogen after adding adjuvants and before inoculation and after fermentation for 7 days. Other symbols have the same meaning as before.

Table 4. *Sporidesmin isolated after growth of Pithomyces chartarum on media from different plant sources*

Batch no.	Medium	N (% dry wt.)	Yield of sporidesmin (mg./100 g. dry medium)
63A	Potato + carrot	1.8	2.6
60	Corn steep liquor*	2.64	0.85
104	Rye corn	1.95	12.9
108	Bran	3.15	19.6

Batches 104 and 108 were 28-day fermentations. Similar results were obtained in numerous repeat experiments.

* Corn steep liquor, obtained from Messrs Clifford Love and Co., Sydney, Australia, was collected as the boat berthed and was used the same day.

Semi-quantitative, two dimensional, paper chromatography (kindly carried out by Mr G. R. Russell) of the amino acids present in lyophilically dried samples of culture filtrates taken at 2, 3, 5 and 7 days after inoculation showed a steady decrease in the quantities of these substances present. As glutamine appeared to be utilized more rapidly than the other amino acids it was chosen as a source of organic nitrogen. Again most of the nitrogen added remained in the solution at the end of the fermentation (Table 3). Increased growth was observed but also yields of sporidesmin and the sporidesmolides were increased almost in proportion to the quantity of glutamine added. The experiments relating to the addition of glutamine summarized in Table 3 were carried out with a different strain of the organism from that (strain C) used in the rest of this work. This strain (see Methods) produced

about four times as much sporidesmin when grown under exactly the same cultural conditions as did strain C. Thus a two-fold increase in sporidesmin production, on addition of 0.1% (w/v) glutamine, was more convincing in the case of the former strain because of the limits of accuracy of the analytical methods.

These results suggested that higher yields of sporidesmin were associated with rich starting medium containing glutamine, so the growth of the fungus and its production of sporidesmin and sporidesmolides was investigated on wet grain and bran.

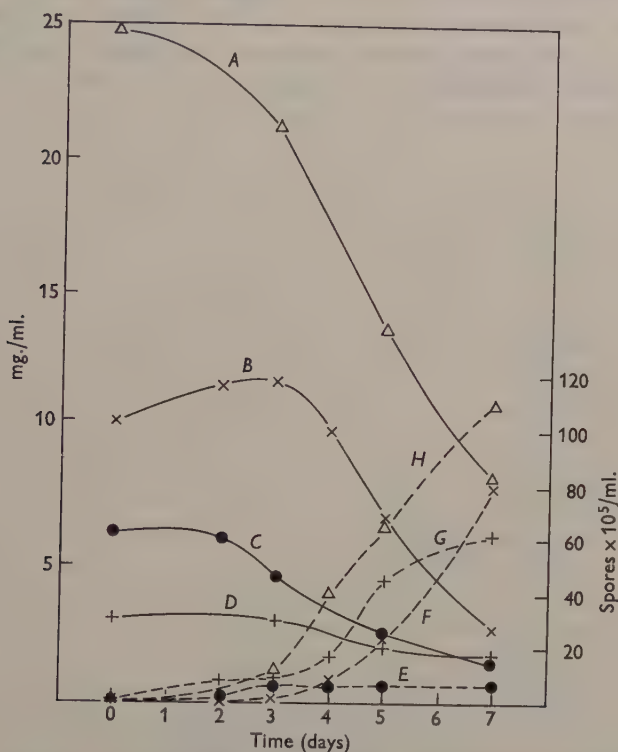


Fig. 2. Utilization of medium constituents and production of sporidesmin and sporidesmolides as a function of culture age. *A*, Dry weight of media; *B*, sugar; *C*, nitrogen ($\times 10$); *D*, ash; *G*, mycelium dry weight; *H*, spore count; *F*, sporidesmolides ($\times 100$); *E*, sporidesmin ($\times 1000$).

Lloyd & Clarke (1959) showed that *Pithomyces chartarum* grows well on rye grain (*Secale cereale*) and this has been confirmed in this work. Considerable precautions were required to make sure that residual contaminants were absent after autoclaving. Table 4 compares the yield obtained after culture on rye grain and bran with that obtained on potato carrot medium and corn steep liquor.

Figure 2 summarizes results relating to the change with time of medium dry weight, medium ash values, sugars, nitrogen, fungal growth and spore, sporidesmin and sporidesmolide formation obtained partly from about 120 batches but particularly from six of these batches in which all the analytical data were obtained. The apparent rise in sugar concentration (determined iodometrically) in the first 2-3 days of the fermentation has been noted frequently. In the absence of ammonium salts

the pH value of the medium increased by more than 2 units in a 7 day fermentation. It was found that this gave a good indication of the growth of a culture. The addition of ammonium salts had a buffering effect; changes of only 0.5–1 unit were observed in this case.

Only traces of sporidesmin were isolated from cultures less than 72 hr. old. After this time the quantity of this metabolite isolated from a given batch did not vary within the limits of accuracy of the analytical methods used. Thus sporidesmin appears in the fermentation at the start of vigorous growth.

The weight of sporidesmolides isolated from unit volume of culture medium is plotted in Fig. 3 as a function of spore numbers in the same volume. Two relations are apparent. Thus the linearity of the relation is independent of the time of fermentation and of the particular experiment.

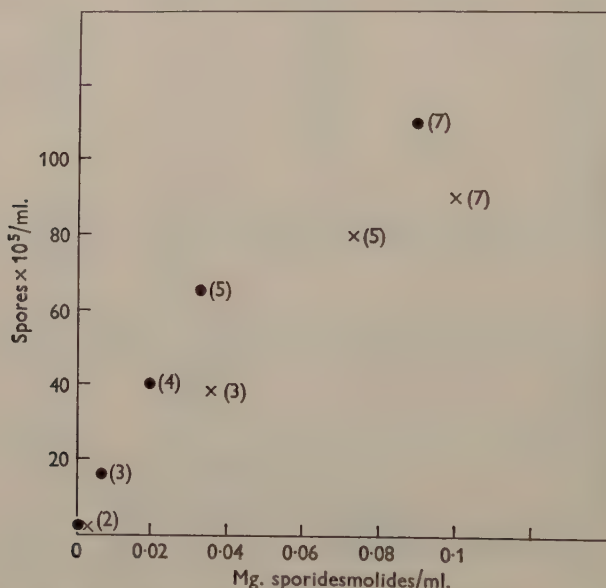


Fig. 3. Spore count/ml. medium as a function of sporidesmolide production by *Pithomyces chartarum*. ● ●, Batch no. 111; × ×, batch no. 118. Figures in parentheses indicate the age of culture (days) at which the particular analysis was made.

DISCUSSION

At the beginning of this work, it was clear that the production of sporidesmin by *Pithomyces chartarum* growing on potato carrot medium was poor. Antibacterial bioassay could not be used and other methods had to be found. A cytopathological effect of extracts of *P. chartarum* on cultures of HeLa cells was demonstrated by Murphy & Worker (1960). Evidence that this cytopathological effect was specifically due to sporidesmin was obtained by an examination of least toxic dose values on alternate fractions from the reversed phase partition chromatogram of Synge & White (1959). Perfect correlation between the highest dilution exhibiting the least toxic dose and a maximum in fraction weight was observed. Side fractions were inactive. The effective concentration of sporidesmin for HeLa cells was about

10^7 molecules/cell; this is about the lowest effective cytopathological concentration of the more cytotoxic compounds (colchicine and actinomycin D) studied by Eagle & Foley (1958).

The application of tissue culture toxicity tests to the examination of many samples from a production programme is severely restricted on a small budget. Recently (Done, Mortimer & Taylor, 1960) the inflammatory nature of the changes found in the livers of sheep showing clinical facial eczema has been emphasized. It seemed that these phenomena might be due to the presence of a toxin ingested from pasture. Further speculation associated this toxin with sporidesmin. It therefore seemed pertinent to investigate the possibility that inflammatory changes could be induced by extracts containing this material on tissues other than the liver. Such extracts were therefore instilled into rabbit's eyes and were shown to initiate the changes described in the Methods section and shown in Plate 1. Larger amounts than those described in the assay procedure cause correspondingly more severe inflammatory changes which also develop more rapidly. At the highest dose used (40 μ g.) severe oedema was seen 36 hr. after the second instillation and a severe keratitis and ulceration followed with the formation of a sedimented layer of leucocytes in the anterior chamber of the eye. This procedure was used in the earliest stages of this work, before crystalline sporidesmin was available, and proved useful for comparing the biological potency of materials from different sources. It was simple to carry out, and later the results showed good agreement with those obtained by the tissue culture toxicity test and by the iodometric assay.

The possibility was considered that a limiting factor in the formation of sporidesmin by *Pithomyces chartarum* was the early exhaustion from the medium of a specific precursor of this metabolite. The fermentation displays some similarities to penicillin fermentations and the change in pH value of the medium suggested that a precursor might be acidic. However extracts of acidified starting medium did not increase the isolated yield of sporidesmin from cultures to which the extracts had been added. An explanation of this negative result might be that the postulated precursor is water soluble and not extractable under the conditions used. The effect of glutamine might be support for this view; however, the latter effect may be analogous to the effect of methionine in the production of cephalosporin N by *Cephalosporium acremonium* (C.M.I. Herbarium no. 49,137; Miller, Kelly & Newton, 1956).

The number of examples of macrocyclic peptide-like substances isolated from cultures of bacteria and fungi is rapidly increasing. In the case of bacteria the compounds are usually true cyclic peptides, e.g. polymyxin B₁ (Hausmann, 1956; Biserte & Dautrevaux, 1957). One example of this group, bacitracin, has been shown to be closely associated with the sporulation process of *Bacillus licheniformis* (Bernlohr & Novelli, 1960). Two metabolic products, valinomycin (Brockmann & Geeren, 1957) and amidomycin (Vining & Taber, 1957) have been isolated from species of *Streptomyces*; these compounds are macrocycles consisting of eight units of alternative α -hydroxy- and α -amino acids. The similarity of these compounds to the enniatins studied by Plattner and his co-workers (see, for example, Plattner & Clauson-Kaas, 1945; Plattner & Nager, 1948) was pointed out by Young (1957). This group of compounds occurs widely in *Fusarium* spp. (Lacey, 1950) and they are, structurally, closely related to the sporidesmolides (Russell, 1960). The

results in Fig. 3 support the hypothesis (Russell & Brown, 1960) that the sporidesmolides behave as an impermeable conidial coat. In view of the structural relationship of the whole of this group of substances it seems possible that they may behave generally in this way.

The practical importance of predicting the toxicity of pasture and the elucidation of the possible role of the fungus in connexion with facial eczema perhaps excuse speculation on the application of the results reported in this paper to the understanding of the growth of the organism in the field. It is widely held that pastures become toxic to grazing animals when pasture plants are growing rapidly (see, for example, Filmer, 1958). Should these conditions favour rapid growth of the fungus the results in Fig. 2 suggest that sporidesmin is produced at the onset. Thus techniques such as field counting of spores are unlikely to be of use in forecasting an outbreak of the disease since high spore counts will post-date the onset of toxicity in a pasture. The production of sporidesmin on four different undefined (Table 4) and defined (Ross, 1960) media also suggests that changes in the common species present in pasture will not affect the growth of the organism nor its ability to produce sporidesmin.

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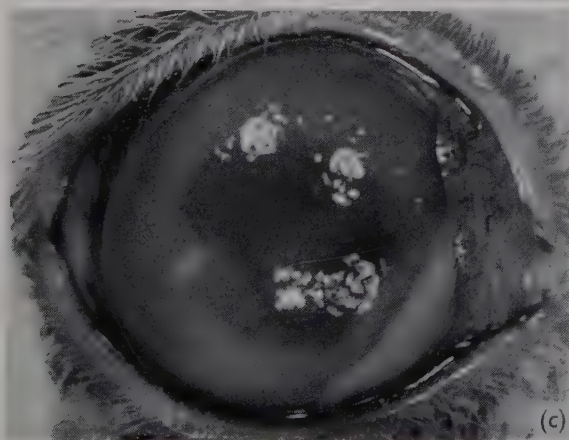
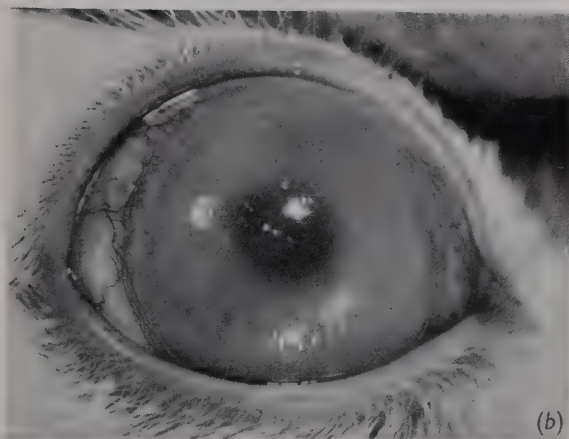
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EXPLANATION OF PLATE 1

(a) A normal rabbit's eye.

(b) Eye showing congestion and proliferation of blood vessels at the corneo-scleral junction. The cornea shows obvious opacity due to migrating leucocytes in its substance. This type of lesion was produced by the instillation of 20 μ g. sporidesmin into the conjunctival sac.

(c) Eye showing changes described in (b) but with more severe corneal opacity. Note especially the white layer of leucocytes which is present in the anterior chamber, having sedimented down at the inner (lower) canthus of the eye. The *membrana nictitans* is seen to be inflamed and oedematous. This severe eye lesion was produced by the instillation of 40 μ g. sporidesmin. Plate 1 (a) and Pl. 1 (c) are respectively the left and right eye of the same animal.



Growth of *Leptospira* in Defined Media

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SUMMARY

Serum-free chemically defined media were developed for cultivating several *Leptospira* strains. *Leptospira canicola*, Ruebeusch strain, was the main test strain. Esterified fatty acids (monoolein, monostearin, methyl palmitate, methyl oleate) replaced serum. Fe^{++} over a narrow range replaced haemin. Thiamine was essential. Other vitamins added to thiamine-containing medium (nicotinic acid, pantothenate, putrescine, *p*-hydroxybenzoic acid, vitamin B_{12}) speeded or increased final growth. Acetate stimulated growth moderately. Ammonium sulphate was favourable over a wide concentration range.

INTRODUCTION

Many investigators (e.g. Woratz, 1955; Fulton & Spooner, 1956; Alston & Broom, 1958; Broom, 1959) have noted that leptospires generally require serum or serum fractions for growth; nevertheless, Savino & Rennella (1944) and Woratz (1957) serially transferred *Leptospira icterohaemorrhagiae* and *L. canicola* without serum or serum fractions. Woratz's medium contained gelatin and a tryptic digest of casein. Savino & Rennella could grow only 4 of their 12 strains in a defined medium containing 'activators'—a mixture of vitamins (nicotinic acid, nicotinamide, thiamine, pyridoxine, riboflavin, pimelic acid) and aspartic acid. The present paper describes a chemically defined medium which permits continued serial growth of various leptospires.

METHODS

Leptospira canicola, Ruebeusch strain, was the initial experimental organism. This strain, originally carried in Korthof medium (Alston & Broom, 1958) containing 10% (v/v) pooled rabbit serum, was transferred to agar-free basal medium (BM-AF, Table 1) containing 1% (v/v) serum. This culture served as inoculum for preliminary experiments. A standard loopful (3 mm. diam.) covered with a 10 mm. \times 10 mm. coverglass had about 6 leptospires/high-dry dark field (\times 440) after incubation for 7-14 days at 30°.

For counts by dark field, 0.01 ml. of culture was put on a slide and covered with a 22 mm. coverglass. As determined by ocular micrometer, the area of a high-power (\times 530) field was 0.0962 mm.² The number of fields per coverglass was 484 mm². (area of the coverglass)/0.096 = 5040. The count/ml. was thus obtained by multiplying the count per high-power field by 5040 \times 100. Usually 20 fields were used for

a count, and duplicate counts agreed to within 10 %. With sparse cultures counting was done at a $\times 100$ magnification.

The same concentration of serum in BM medium (containing 0.1 % agar) permitted faint growth after 2 weeks as a faint line about 20 mm. below the surface (Dinger's phenomenon: Fletcher, 1928; Lawrence, 1951; Czekalowski, McLeod & Rodican, 1954). Serum at 0.1 % (v/v) did not give visible growth in 2-3 weeks.

Cultures of *Leptospira canicola* intended as inocula were transferred serially in BM-AF medium containing 1 % (v/v) serum. The initial nutritional experiments were undertaken in BM medium containing 1 % (v/v) serum.

The ingredients required for 10 ml. of final medium were dissolved in 9 ml.; the inoculum brought it to 10 ml. Inoculation was by means of a Cornwall side-arm syringe (Becton-Dickenson, B-D, no. 1251) delivering 1 ml. through a short 15-gauge needle passing through a Pyrex filling attachment (Corning No. 3960). Connexions were made between the syringe and needle by means of B-D adapters H 468/L and L/606 inserted in 3 mm. diam. gum-rubber tubing. The inside delivery tip of the attachment was filed back to allow the needle to project freely. Inoculation was made after the autoclaved medium had cooled to about 37°, before solidification of the agar, and was forceful enough to mix the contents of the tube.

It was convenient to prepare BM medium 5-fold concentration without agar. The pH value was adjusted with Quadrol (*N,N,N',N'*-tetrakis (2-hydroxypropyl)-ethylenediamine; Wyandotte Chemicals Corp., Wyandotte, Mich., U.S.A.; Hutner, Cury & Baker, 1958), a few drops of volatile preservative added (Hutner *et al.* 1958), and the concentrate stored in the refrigerator. Test substances were prepared in ten-fold concentration and preserved similarly.

Since distillates from bleached cotton can inhibit fatty acid-sensitive organisms (Drea, 1946), the $\times 16$ 150 mm. Pyrex culture tubes were capped with inverted shell vials. Later experiments were made in 50 ml. screw-cap flasks containing 20 ml. medium. Media were autoclaved at 121° for 15 min.

RESULTS

As growth-promoters were identified, a serum-free but complex medium was assembled (Medium 283, Table 1) which supported serial growth of *Leptospira canicola*, also *L. pomona* L-9 and *L. icterohaemorrhagiae* N.I.H. strain. Dissection of medium 283 to identify its active components led to several defined agar-free media (Table 1) which supported the growth of the three original strains and 9 of 11 additional strains of *Leptospira* (Table 2). The point of departure in developing the defined medium was the observation that a supplement of 1 % (v/v) serum permitted growth of *L. canicola*, Ruebeusch strain, in BM and BM-AF media; 0.1 % (v/v) serum was ineffective.

Proteose peptone (Difco) increased growth in the presence of 1 % (v/v) serum but did not replace serum. Liver fraction 'L' (Nutritional Biochemicals Corp., Cleveland 28, Ohio, U.S.A.), soluble starch and haemin were inert or inhibitory at higher concentrations.

The Fe:Co ratio was critical; Table 3 shows a typical experiment.

The three original test strains did not grow as well in defined media as in Korthof medium containing 10 % (v/v) serum; growth however did exceed that obtained

with 1% (v/v) serum; many strains (Table 4) grew well although less than in Korthof medium with 10% (v/v) serum.

In serum-free medium, 0.5% (v/v) of a blunderbuss mixture consisting of an acid-hydrolysate of gelatin, L-tryptophan, DL-methionine, alkali-hydrolysed yeast nucleic acid, acid-hydrolysed deoxyribonucleic acid and vitamins (complete

Table 1. *Serum-free and chemically defined media for leptospirae*

Media were adjusted to final pH 7.4-7.5. Sterilization was by autoclaving at 121° for 15 min.

Quantities as mg. or ml./100 ml. final medium.

	Basal medium (BM)	Transfer medium no. 283	Serum-free medium no. 198 A	Chem.-def. medium no. 198 B	Chem.-def. medium no. 198 E	Chem.-def. medium no. 198 F
KH ₂ PO ₄	10	10	10	10	10	10
MgSO ₄ ·7H ₂ O	40	40	40	40	40	40
Ca ⁺⁺ (as Cl ⁻)*	0.4	0.4	0.4	0.4	0.4	0.4
Agar, Special† (Noble, Difco)	100	100
HEDTA‡	.	2.0	2.0	2.0	2.0	2.0
TEM-4T§	.	3.5	3.5	.	.	.
Hycase SF	.	10
'Complete suppl. No. 9'	.	0.5 ml.
'Metals 45A'	.	1.2 ml.
Fe ⁺⁺	.	.	0.1	0.1	0.1	0.1
Mn ⁺⁺	.	.	0.015	0.015	0.015	0.015
Zn ⁺⁺	.	.	0.15	0.15	0.15	0.15
Cu ⁺⁺	.	.	0.008	0.01	0.01	0.01
Na acetate. 3H ₂ O	.	.	5.0	5.0	5.0	5.0
Co ⁺⁺	.	.	0.0015	0.01	0.01	0.01
(NH ₄) ₂ SO ₄	.	.	.	20	20	20
Nicotinic acid	.	.	.	0.02	0.02	0.02
Thiamine HCl	.	.	0.04	0.04	0.04	0.04
Ca pantothenate	.	.	.	0.02	0.02	0.02
Pyridoxamine. HCl	.	.	.	0.02	0.02	0.02
Vitamin B ₁₂	.	.	.	0.00002	0.00002	0.00002
DL-Alanine	10	.
DL-Aspartic acid	50	.
DL-Isoleucine	.	.	.	1.0	1.0	.
DL-Leucine	.	.	.	1.0	1.0	.
DL-Methionine	0.5	.
DL-Phenylalanine	.	.	.	5.0	5.0	.
DL-Serine	1.0	.
DL-Tryptophan	2.0	.
DL-Valine	5.0	.
Glycine	1.0	.
L-Glutamic acid	10.0	.
L-Histidine HCl. H ₂ O	25	.
L-Proline	2.0	.
Monocolein	.	.	.	2.0	2.0	2.0

* Ca was weighed as CaCO₃ and dissolved in dilute HCl.

† Agar may be eliminated = BM - AF.

‡ HEDTA = hydroxyethylethylenediamine-triacetic acid.

§ Supplied by Hachmeister Inc., P.O. Box 357, Pittsburgh, 30, Pa., U.S.A. TEM-4T is a mixture of diacetyl tartaric acid esters of glycerides from tallow.

|| Hycase SF = 'low-salt' acid-hydrolysed casein, Sheffield Chemical Co., Norwich, New York, U.S.A.

supplement No. 9; Hutner *et al.* 1957), supported growth provided that a semi-synthetic fat, TEM-4T (Shorb & Lund, 1959; a mixture of diacetyl tartaric acid esters of glycerides from tallow) was present. Addition of Hycase SF (Sheffield Chemical Co., Norwich, N.Y., U.S.A.), a 'salt-free' acid hydrolysate of casein, increased growth further (Table 5). Of the components of the 'complete supplement', the alkali-hydrolysed yeast nucleic acid had a slight effect, the vitamin mixture somewhat more.

Absolute requirements; lipids and vitamins. Thiamine was essential. Supplementation with nicotinic acid or pantothenate speeded growth. Putrescine also was

Table 2. *Strains of Leptospira investigated*

	Designation	Culture no.
<i>L. autumnalis</i>	Fort Bragg	5287*
<i>L. ballum</i>	Garcia	5303*
<i>L. bataviae</i>	EER (AM 2)	5304*
<i>L. canicola</i>	Ruebeusch	RU 43†
	Utrecht	5937*
	Undesignated	39660*
<i>L. grippotyphosa</i>	Andaman CH 31	5862*
<i>L. icterohaemorrhagiae</i>	Wijnberg	5309*
	Undesignated	39661*
	M 20	5938*
	NIH	NIH†
<i>L. pomona</i>	Johnson	5939
	Undesignated	L-9†

* Supplied through the courtesy of Dr A. H. Harris and Julia M. Coffey, Div. Labs., N.Y. State Department of Health. See *Annual Rep. Div. Laboratories and Research*, 1953, New York State Department of Health, Albany, N.Y., p. 149, 'Collection of Type Cultures'.

† Original strains from Communicable Disease Center, U.S. Public Health Service, Chamblee, Georgia, U.S.A.

Table 3. *Iron-cobalt relations for growth of Leptospira canicola (Ruebeusch strain)*

Medium: basal medium (100 ml.) + TEM-4T*, 3.5 mg. + HEDTA† 2 mg. + 'vitamin mix No. 12', 0.002 ml.; agar ('Noble', Difco, 0.1 %, w/v); pH 7.4-7.5. Fe⁺⁺ was added as Fe(NH₄)₂(SO₄)₂·6H₂O. Growth recorded after incubation for 14 days at 30°. Arbitrary scale: 0 = no growth; + + + + = growth as in Korthof + 10 % (v/v) serum medium. Quantities of Fe⁺⁺ and Co⁺⁺ are in mg./100 ml. medium.

Fe ⁺⁺ (mg.)	Co ⁺⁺ (mg.)				
	No addition	0.0125	0.025	0.05	0.10
	Relative growth				
No addition	0	0	0	0	0
0.031	0	0	0	0	0
0.062	0	0	0	0	0
0.12	++	++	++	++	++
0.25	++	++	+	+	0
0.5	0	+	+	0	0
1.0	0	0	0	0	0

* TEM-4T = a mixture of diacetyl tartaric acid and esters of glycerides of tallow (see Table 1).

† HEDTA = hydroxyethylethylenediamine-triacetic acid.

favourable but not so clearly as was nicotinic and pantothenic acids; *p*-hydroxybenzoic acid and vitamin B₁₂ also stimulated slightly.

In the presence of 1% (v/v) serum, the optimum concentration of TEM-4T was 3.5 mg./100 ml.; Tween 80 supported growth from about 1 to 80 mg./100 ml. Tweens 60 and 40 were less satisfactory. Cholesterol at 0.5–4 mg./100 ml. and soybean lecithin from 0.6–5 mg./100 ml. were without effect in the presence of serum and did not replace serum.

In serum-free media, monacetin, methyl linoleate, and methyl linolenate at 0.5–

Table 4. *Growth responses of Leptospira strains in serum-free chemically defined media (198B, 198E, 283; see Table 1); and in Korthof medium + 10% (v/v) pooled rabbit serum*

Flasks inoculated with 10⁵ leptospirae/ml. Most inocula made from cultures in 11–15th transfer except *L. canicola* no. 5937 (6th transfer). *L. canicola* (Ruebeusch) inoculation made from culture in 38th transfer; in medium 283; *L. pomona* (L-9) and *L. icterohaemorrhagiae* (N.I.H.) from 28th transfer.

<i>Leptospira</i> spp. species and strain	Defined medium							
	198B		198E		283		Korthof 10% (v/v) rabbit serum	
	Max. growth (leptospirae × 10 ⁻⁶ /ml.)		Max. growth (leptospirae × 10 ⁻⁶ ml.)		Max. growth (leptospirae × 10 ⁻⁶ ml.)		Max. growth (leptospirae × 10 ⁻⁶ ml.)	
	Days		Days		Days		Days	
<i>L. bataviae</i> no. 5304	14	40	10	120	.	.	10	360
<i>L. canicola</i> no. 39660	10	85	14	44	.	.	17	140
<i>L. canicola</i> no. 5937	14	65	7	35	.	.	10	220
<i>L. canicola</i> Ruebeusch	14	25	.	.	7	10	10	610
<i>L. grippityphosa</i> no. 5862	.	.	10	100	.	.	10	600
<i>L. icterohaemorrhagiae</i> no. 5938	14	45	10	66	.	.	14	360
<i>L. icterohaemorrhagiae</i> no. 39661	10	100	10	80	.	.	10	170
<i>L. icterohaemorrhagiae</i> N.I.H.	7	60	.	.	7	10	10	600
<i>L. pomona</i> No. 5939	17	33	14	92	.	.	17	800
<i>L. pomona</i> L-9	14	.	17	1200

Table 5. *Hycase SF*/TEM-4T† relationships for growth of Leptospira canicola*

Basal medium: HEDTA 2 mg./100 ml.; metals 45A 1.2 mg./100 ml.; 'complete supplement no. 9', 0.5 ml./100 ml., 'Noble' agar 0.1% (w/v); pH 7.4–7.5.

TEM-4T (mg./100 ml.)	Hycase conc. (mg./100 ml.)				
	No addition	40	20	10	5
10	+	+	±	±	±
5	±	++	++	++	+
2.5	0	0	±	±	+
1.25	0	0	0	0	±

* Hycase SF = low-salt acid hydrolysed casein (see Table 1).

† TEM-4T = mixture of diacetyl tartaric acid esters of glycerides of tallow (see Table 1).

8 mg./100 ml. were unsatisfactory fatty-acid sources. Monoolein, methyl oleate, methyl palmitate or monostearin could replace TEM-4T; monoolein was best. A mixture of 2 mg. each of monoolein, monostearin and methyl palmitate/100 ml. allowed earlier but not consistently heavier growth.

Table 6. *Serial transfer of Leptospira strains in serum-free chemically defined media*

Inoculum size†	Media									
	198 A		198 B		198 E		198 F		283	283 SS*
	10 %	50 %	10 %	50 %	10 %	50 %	10 %	50 %	10 %	10 %
Species	No. of successful transfers									
<i>L. autumnalis</i> no. 5287	7‡	19	.	23	.	3‡	12‡	23	.	.
<i>L. ballum</i> no. 5303	.	2‡	.	2‡	.	3‡	.	1‡	.	.
<i>L. bataviae</i> no. 5304	21‡	21	25	24	16	.	6‡	18	.	.
<i>L. canicola</i> no. 39660	17	18	25	23	21	21	14‡	23	.	.
<i>L. canicola</i> no. 5937	22	19	1‡	7‡	10	.	1‡	10	.	.
<i>L. canicola</i> (Ruebeusch)	22	23	25	24	1‡	14	20	23	38	39
<i>L. grippotyphosa</i> no. 5862	16‡	17‡	4‡	11	19	15	22	19	.	.
<i>L. icterohaemorrhagiae</i> no. 5938	16‡	14‡	23	24	23	19	9‡	23	.	.
<i>L. icterohaemorrhagiae</i> no. 3228	17	22	.	4‡	.	4‡	.	4‡	.	.
<i>L. icterohaemorrhagiae</i> no. 39661	.	18	22	24	21	19	22	25	.	.
<i>L. icterohaemorrhagiae</i> no. 5309	.	4‡	.	3‡	.	3‡	.	5‡	.	.
<i>L. icterohaemorrhagiae</i> (N.I.H.)	14	25	18	.	1‡	3‡	17	18	29	.
<i>L. pomona</i> no. 5939	20	22	25	22	22	18	13‡	23	.	.
<i>L. pomona</i> (L-9)	6	23	.	3‡	.	3‡	17	13	29	.

* Semi-solid medium.

† Inoculum size; % values refer to volume of culture transferred to volume fresh medium.

‡ Died.

Amino acids. The values given in this section are mg. compound/100 ml. medium. Slight growth stimulation was obtained with glycine (0.8–3 mg.), DL-alanine or L-glutamic acid (12.5–50 mg.), L-lysine HCl or DL-methionine (1.6–6.25 mg.), L-proline or DL-valine (2.5–20 mg.), and DL-aspartic acid (50–100 mg.). Moderate stimulation was obtained by L-histidine (free base) (12.5–50 mg.), DL-isoleucine (1.2–5.0 mg.), DL-leucine (0.65–2.5 mg.), DL-serine (0.008–0.125 mg.), and DL-tryptophan (1.25–10 mg.). Marked stimulation was obtained with DL-phenylalanine (0.6–20 mg.). L-Arginine HCl (0.4–3.1 mg.), DL-threonine (0.078–2.5 mg.), and L-tyrosine (0.078–0.156 mg.) were inert.

A mixture of metals '45A' (Hutner *et al.* 1957) supported growth. Fe⁺⁺ alone supported growth only in a narrow range (0.125–0.5 mg). Hycase became unnecessary for growth with adequate Fe⁺⁺. Individually added to Fe, Co (0.001–0.04 mg.), Cu (0.0015–0.24 mg.), Mn (0.015–0.02 mg.), or Zn (0.005–0.34 mg.) augmented growth. Since the metal requirements were critical, metal-buffering with the chelating agent of hydroxyethylethylenediamine-triacetic acid (HEDTA;

Provasoli & Pintner, 1960) was inhibitory at low concentrations of serum or TEM-4T. HEDTA 2 mg. was optimal in the presence of low concentrations of serum or in chemically defined media containing the standard metal mixture (Table 1). Acetate was moderately stimulating. Ammonium sulphate increased growth remarkably over a very wide range (1.95–125 mg.). Table 4 shows the growth responses of various leptospiral strains in the experimental media developed. Table 6 indicates the number of successful transfers made when either an equal volume of inoculum was added to fresh media or a 1:10 ratio of inoculum to fresh medium was used.

DISCUSSION

Previous workers were unable to replace serum with amino acids and vitamins (Chang, 1947 *a, b*; Green, Camien & Dunn, 1950), or synthetic ion-exchange resins, charcoal or polyvinylpyrrolidone (Marshall, 1949; Fulton & Spooner, 1956; Johnson & Wilson, 1960). That lipid requirements were the major obstacle had been foreshadowed; defatted fraction V of blood serum no longer stimulated respiration of *Leptospira icterohaemorrhagiae* (Helprin & Hiatt, 1957) or supported growth of treponemes (Oyama, Steinman & Eagle, 1953). Our work confirms that of Woratz (1957) and Ivler (1960) that oleic acid esters promote growth of *Leptospira*. As their media had complex natural materials, one could not tell whether the fatty-acid requirement was absolute. A close parallel to our results are those of Power & Pelczar (1959), who found TEM-4T an advantageous source of essential fatty acids for the Reiter treponeme; a mixture of palmitic, stearic and oleic acids permitted the same degree of growth.

That the Fe concentration is critical probably accounts for the seeming dependence of these organisms on compounds such as haemoglobin. Strains not having catalase may appear to need exogenous haeme. Fe seems adequate for strains with catalase. Faine (1960) found appreciable amounts of catalase in certain pathogenic *Leptospira* strains. The optimum concentration of Fe for *Leptospira canicola* agrees well with that reported by Faine (1959) for *L. icterohaemorrhagiae*.

Thiamine, found stimulatory by others, e.g. Savino & Rennella (1944), may well be an absolute requirement for all leptospires.

The results with amino acids obtained by other investigators are difficult to compare with ours; different basal media were used, the presence of serum complicates matters, and the purity of amino acids has improved in recent years. Growth of a wide range of strains in chemically defined media was achieved here without introducing new factors, merely by applying information scattered in the literature. Whether strains which will not grow in the present defined media require additional growth factors or are exceptionally sensitive to nutritional imbalances remains to be decided. Another problem is to improve defined media so that they permit growth equal to or greater than that in media which contain complex natural materials such as serum.

More specific, more conveniently prepared antigens for diagnosis of leptospiral diseases are needed as recognition grows of the gravity and economic importance of these infections in animals and man. The availability of defined media may lead to better media for diagnosis by direct cultivation and to practical antigen-free media for the preparation of diagnostic antigens.

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Distribution of Urease in *Clostridium perfringens* Types

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SUMMARY

About five hundred strains of *Clostridium perfringens* were tested for ability to produce urease. There was found to be a distinct, although not complete, correlation between urease production and antigenic type. In types B and D there was also some degree of correlation between the production of urease and of λ -antigen. The distribution of urease between the six antigenic types of *C. perfringens* was not sufficiently sharp to be of use in type differentiation.

INTRODUCTION

Clostridium perfringens has been subdivided into six types: A to E distinguished by the production of one or more of the major lethal toxins α , β , ϵ and ι ; and F, which on the basis of toxin production would be included in type C, distinguished mainly by heat resistance, large cell size and epidemiology (Wilsdon, 1931; Glenny *et al.* 1933; Oakley, Warrack & Warren, 1948; Oakley, 1949; Ross, Warren & Barnes, 1949; Zeissler & Rassfeld-Sternberg, 1949). Each of these types, including F, is further characterized by the production of a number of minor antigens, enzymic in nature, and distinguished by their effects on appropriate substrates and by the neutralization of these effects by the corresponding type antisera. In the course of a general survey of the cultural and biochemical characteristics of various *Clostridium* spp. a number of *C. perfringens* strains was examined for ability to produce urease. Since some of these gave positive and others negative results, a large number of strains was examined to determine whether any correlation existed between urease production and antigenic type. The results of this survey, together with the results of experiments attempting to elucidate the nature of *C. perfringens* urease, are reported in this paper.

METHODS

Strains examined. Four hundred and ninety-two strains were examined, divided between types as follows: type A, 267 strains; type B, 63; type C, 64; type D, 68; type E, 10; and type F, 20 strains. Each of the ecological subtypes recognized within types B and C was represented (Brooks, Sterne & Warrack, 1957), and the strains were drawn from a wide range of habitats, geographically widespread and including pathological and non-pathological sources. Classical and vaccine-production strains and freeze-dried and freshly isolated cultures were included in the survey.

Preparation of test material. Fifteen ml. amounts of Robertson's cooked meat medium in one ounce screw-cap bottles were inoculated from broth cultures, or

with colonies picked from the surface of blood-agar plates, and incubated at 37° for 18–24 hr. Some strains were also grown in 57 ml. amounts of a similar medium, and samples tested after 5, 24, 48 and 72 hr. incubation.

Duplicate 3.0 ml. samples from well-grown cultures were placed in small glass tubes and centrifuged. The supernatant fluids were discarded and the sediments washed once in 1.0 % (w/v) saline. The washed sediments were used for testing without any further processing or addition of preservatives. Some samples were given up to five further washings with 1.0 % saline but in no case did this alter the final result of the test, and it was therefore concluded that the single washing was adequate.

Reagents. The reagent used for routine testing (solution A) was a modified version of that used by Ferguson & Hook (1943) and by Anderson (1945), and was made up as follows: 0.1 g. KH_2PO_4 ; 0.1 g. K_2HPO_4 ; 0.5 g. NaCl; 2.0 g. urea; 0.5 g. phenol; 1.0 ml. 95 % (v/v) ethanol in water; 100 ml. distilled water; 5.0 ml. Universal Indicator (British Drug Houses Ltd., Poole); and sufficient 0.1 N-HCl to give an orange colour (about pH 6.0).

A control reagent (solution B) in which the urea was replaced by a further 2.0 g. NaCl was made up, and also both test and control reagents as above but omitting the Universal Indicator (solutions C and D, respectively).

Procedure for urease tests. For the routine test one of each pair of culture sediments was resuspended in 2.0 ml. of solution A and the other in the same volume of solution B, and both suspensions were incubated at 37° for 18 hr. The development of alkalinity, as indicated by a colour change to green or blue, in the solution A tube only, was taken to indicate the presence of urease. None of the strains examined gave a positive reaction with solution B. Although the results of the test were not recorded until 18 hr. incubation, the colour change indicating the presence of urease developed within minutes in strongly urease-positive cultures and was evident in all positive suspensions after 2–4 hr. at 37°. Some tests showing negative results were incubated for up to 72 hr. but still showed no tendency to develop alkaline reactions.

For the Nessler's reagent test the sediments were resuspended in solutions C and D, respectively, and incubated at 37°. Samples were removed after 18 hr. and tested for the presence of NH_4^+ by the addition of Nessler's reagent. The appearance of an orange-yellow precipitate, in the solution C test only, was taken to indicate urease production. No strain gave a precipitate with solution D.

Suspensions of cultures subjected to ultrasonic disintegration were tested with both the routine and the Nessler's reagents. Equal volumes of suspension and reagent were incubated together at 37° for 18 hr. and the results interpreted as described above.

Preparation of cell suspensions for ultrasonic disintegration. Seventy-five ml. amounts of a broth containing meat particles were inoculated and incubated at 37° for 18 hr. After the meat particles had been strained off, the broths were centrifuged, the supernatant fluids discarded and the sediments washed in phosphate buffer (pH 7.3) and finally resuspended in 10 ml. amounts of the same buffer. After the removal of a 2.0 ml. sample the remainder of each suspension was subjected to ultrasonic disintegration for 10 min. (Mullard 500 watt E7590-B disintegrator).

Preparation of urease suspensions for titration of anti-urease activity. Urease-

positive cultures were grown for 18–24 hr. in 75 ml. amounts of meat broth, harvested, centrifuged, and the sediments washed in 1.0 % saline and finally re-suspended in 1.0 % saline. These suspensions proved to be fairly stable and showed little change in urease activity after several days storage at 4.0°.

Two dried urease preparations were obtained by drying washed bacteria harvested from 12 l. cultures of urease-positive type B and type F strains. The washed bacteria were dried in desiccators over silica gel and subsequently finely ground to give homogeneous preparations.

Sera. Antibacterial and antitoxic sera prepared by the hyperimmunization of horses, and antibacterial sera prepared by the hyperimmunization of rabbits, against urease-positive and urease-negative *Clostridium perfringens* strains, were tested for ability to inhibit the urease reaction. Normal horse and rabbit sera were used as controls. All the immune sera contained antibodies to the appropriate soluble antigens of *C. perfringens*. Two antibacterial sera prepared in rabbits against strains of the urease-producing *C. sordellii* were also tested.

Titration of sera for anti-urease activity. One ml. amounts of serial twofold dilutions of the sera under test were mixed thoroughly with 1.0 ml. amounts of suitable dilutions of the fresh or dried urease preparations, and allowed to stand at room temperature for 30 min., after which 0.5 ml. of the routine urease reagent (solution A) was added to each mixture. The results of titrations, as increase in pH value, were recorded after 18 hr. incubation at 37°. Either 1.0 % saline or distilled water was used as diluent, both giving the same results. The pH values were measured with a pH-meter.

The rabbit sera were also titrated using constant volumes of serum and doubling dilutions of the urease preparation.

Test for inhibition of urease activity by urease-negative strains. Fifteen ml. amounts of Robertson's cooked meat broth were inoculated with pairs of the strains under test and incubated at 37° for 24 hr. Smears of each culture were examined, and a culture sample was streaked on a blood agar plate and incubated anaerobically overnight, to determine the presence of growth from both inocula. In the *Clostridium bifermentans*/*C. perfringens* mixtures the examination of smears was sufficient for this purpose. In those *C. perfringens* mixtures where, even on plates, the two strains were indistinguishable, some colonies were picked into saline and tested for urease activity.

RESULTS

Distribution of urease production in Clostridium perfringens types

The results of the urease tests for strains of all types are shown in Table 1. Several strains were tested several times, both as replicate samples from one culture and as replicate cultures from one strain. In all cases the results for any one strain were consistent, even to the extent of giving approximately the same colour change where positive. In cultures incubated for 72 hr. the urease reactions were the same for 5, 24, 48 and 72 hr. samples. About 50 cultures were also tested with Nessler's reagent. All gave results which agreed with those for the routine test.

About one-fifth of the strains examined were found to produce urease, but this property was unevenly distributed between the various types and subtypes. All the type C strains examined were urease-negative. Type A was virtually urease-

negative, having only six urease-producing strains. The urease-positive and urease-negative type A strains were otherwise indistinguishable. Of the six urease-positive strains all were recently isolated, 5 from pathological material and the sixth from the intestines of a normal sheep. Whether or not this can be taken to indicate that type A strains tend to lose the capacity for urease production is doubtful, as numerous freshly isolated type A strains showed no tendency to produce urease. Only 10 type E strains were available for examination, and all of these were urease-positive. Of the 20 type F strains 13 produced urease, and, as in type A, the urease-positive resembled the urease-negative strains in respect of antigen production. The most interesting results were those obtained with the types B and D strains. In both these types there was a distinct correlation between the production of urease and of the λ -antigen (Table 2).

Table 1. *Distribution of urease production in Clostridium perfringens types*

Type	No. strains examined	Urease-positive strains	
		No.	%*
A	267	6	2
B Classical	57	51	89
Iranian	6	0	0
C Classical	31	0	0
Colorado	22	0	0
Piglet	11	0	0
D	68	24	35
E	10	10	100
F	20	13	65

* To nearest 1.0%.

Table 2. *Urease and λ production in types B and D strains*

Type	λ production	No. strains examined	Urease-positive strains	
			No.	%*
B Classical	Unknown	8	8	100
	+ ve	40	37	92.5
	- ve	9	6	67
B Iranian	(all - ve)	6	0	0
D	Unknown	20	4	20
	+ ve	17	16	94
	- ve	31	4	13

* To nearest 0.5%.

Urease and λ production in type B and D strains

The production of λ -antigen is characteristic of the classical type B strains, and is one of the main points of difference between these and the Iranian subtype which is λ -negative. Of the 40 type B strains known to produce λ , 37 produced urease also, whilst only 6 of the 9 λ -negative classical type B strains produced urease. The λ -negative 'Iranian' strains were all urease-negative. One of the urease-negative classical strains was derived from a λ -positive urease-positive parent strain, and had lost the capacity for λ -production also. In type D the correlation

between urease and λ -production was more marked, 16 of the 17 λ -positive and only 4 of the 31 λ -negative strains producing urease.

No similar correlation was observed between urease and the production of any other of the soluble antigens in any of the *Clostridium perfringens* types, except in so far as the major lethal antigens determine type differentiation.

Ultrasonic disintegration

It was thought that the difference between the urease-positive and urease-negative strains might lie not in their ability to produce urease intracellularly, but in the release of the urease from the cells. Bacterial suspensions prepared from positive and negative strains were therefore tested for urease activity before and after ultrasonic disintegration. The urease reactions of the suspensions were in all cases the same before and after disintegration, and agreed with the results of previous tests carried out on the same strains. These results rendered untenable the theory that the differences between strains might lie in the ease of rupture or permeability of the cell walls, rather than in the actual production of urease.

Antigenicity of Clostridium perfringens urease

Some tests were carried out in an attempt to ascertain whether or not the urease produced by *Clostridium perfringens* was antigenic, in the hope that this would show whether the urease was type-specific, species-specific or non-specific. None of the sera tested specifically inhibited the activity of the urease preparations, although several of the sera caused apparent inhibition at low dilutions, presumably by buffering action tending to limit rise in pH value. This effect was observed with normal as well as immune sera. There was thus no evidence that *C. perfringens* urease is antigenic.

Inhibition of urease activity by urease-negative strains

In the course of a study on *Clostridium bifermentans* and *C. sordellii* it was noted that many strains of the urease-negative *C. bifermentans* were able to inhibit the positive urease reactions of *C. sordelli* strains when grown with them as mixed cultures, although this inhibition was not seen when cultures of the two species were mixed after growth (Brooks & Epps, 1959). It seemed possible that the same phenomenon might occur with urease-positive and urease-negative *C. perfringens* cultures. Accordingly 45 mixed cultures of urease-positive and urease-negative *C. perfringens* strains, and two mixed cultures of urease-positive *C. perfringens* and urease-inhibitory *C. bifermentans* were grown and tested for urease activity. In no case was there any evidence of the inhibition of urease activity.

DISCUSSION

The fact that only about 20 % of the strains examined, and only 2.0 % of the type A strains, produced urease probably accounts for the omission of *Clostridium perfringens* from Huet & Aladame's (1952) list of urease-producing anaerobes. Similarly it is not surprising that Ortali & Samarani (1955) found the one *C. perfringens* strain they examined to be urease-negative.

It is unfortunate, from the point of view of pathological investigation, that the

correlation between urease production and type differentiation is not complete. The most that can be said of the urease test as a tool in type differentiation is that since types E and F are rarely encountered, any *Clostridium perfringens* isolate producing urease can probably be assigned to types B or D. The apparent correlation between urease and λ production is similarly unhelpful as a diagnostic tool, but may, in the light of further investigations, help to elucidate the evolutionary relationships of the six *C. perfringens* types. Although the correlation is most marked in types B and D, it is interesting to note that the λ -negative type A was virtually urease-negative, and the λ -negative type C completely so, whilst in the urease-positive type E the five strains examined for λ production were all found to be positive. It is only in the type F strains, with their meagre range of soluble antigens and peculiarly limited distribution (all except one were isolated from cases of necrotic enteritis in Hamburg between 1946 and 1948, and the remaining one was isolated in the same place some years later), that the λ /urease correlation breaks down. No type F strain has been shown to produce λ , although when strains of this type were first isolated they were thought to elicit the production of small quantities of anti- λ in hyperimmune horses and were, therefore, considered to be most nearly related to *C. perfringens* type B (Oakley, 1949). When the question of the position of type F was raised during a survey of *C. perfringens* antigen production, Brooks, Sterne & Warrack (1957) considered type F to be related to type C rather than to type B, on the basis of the range of antigens produced by the three types, and Mrs I. Batty (personal communication) on retesting six of the original type F antisera, found little or no anti- λ in three and only very small amounts comparable to those found in several normal sera, in the other three.

Since it is difficult to see how there can be any direct relationship between urease and λ , particularly since one sometimes occurs without the other, and yet their occurrence together seems too consistent to be purely fortuitous, their distribution may give some indication of the relationships between the various types and subtypes, and the sequence of evolution of the types if a common ancestor is assumed. In this connexion it is of interest that the distribution of urease production between types and subtypes, although not necessarily between individual strains, parallels that of a serum-dependent non- $\alpha\theta\delta$ haemolysis, observed only in cultures grown on blood-agar containing certain antisera (Brooks *et al.* 1957), although as with the soluble λ -antigen, the correlation between the production of urease and of the serum-dependent non- $\alpha\theta\delta$ haemolysis is not complete.

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The Measurement of the Liberation of Penicillinase from *Bacillus subtilis*

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SUMMARY

The formation of penicillinase, induced in a growing culture of *Bacillus subtilis* 6346 by a single addition of benzylpenicillin, spontaneously ceases after about 3 hr. The enzyme continues, however, to be released into the medium, thus permitting the process of its liberation to be studied independently from its production. By using the escape into the medium of a normally intracellular, maltose-inducible α -glucosidase as a more sensitive indicator of cell damage than direct measurement of lysis, it was concluded that at least 40 % of the penicillinase is liberated from the cells without gross disorganization of their structure.

INTRODUCTION

The phenomenon in which certain enzymes are released from apparently undamaged cells of a growing bacterial culture has not so far been the subject of much systematic experimental investigation, the one notable exception being that of the extracellular α -amylase of *Bacillus subtilis*, studied by Nomura, Hosoda & Yoshikawa (1958). In a recent review (Pollock, 1961*b*) some of the principles and definitions involved in studying the problem were evaluated and the difficulties emphasized. There has been considerable scepticism about the reality of exo-enzymes as physiological entities, the view sometimes being expressed that they appear in the extracellular environment only to the extent that the cells are lysed, or damaged to a degree that is incompatible with normal growth and metabolism. However unlikely this view may appear to be in any specific instance, it is not one which can be dismissed without critical experimental evidence to the contrary.

For the work to be described here the penicillinase system of *Bacillus subtilis* NCTC 6346 was chosen for the following reasons.

- (1) The enzyme is stable and can be simply and accurately assayed.
- (2) It is inducible by benzylpenicillin and other closely related compounds, as with the penicillinase of *Bacillus cereus* (Pollock, 1953), and therefore its production can be brought under close control.
- (3) On the reasonable assumption that its mode of production does not differ fundamentally from that of *Bacillus cereus* penicillinase, it could be concluded, on the basis of work done on the latter system (Pollock & Kramer, 1958), that appearance of enzymic activity corresponds to the biosynthesis of the relevant protein molecule itself. No substrate-accessibility factors or high molecular weight precursors appear to be involved to an extent which might complicate interpretation of results based on the direct measurement of enzymic activities.

(4) Unlike the situation in *Bacillus cereus*, however, where 85 to 95% of the penicillinase is extracellular under all conditions so far investigated (Pollock, 1956), the *B. subtilis* enzyme is mainly bound to the cells during the early stages of growth, only later being liberated into the medium. There were some grounds, therefore, for hoping that it might be possible to dissociate liberation of the enzyme from its production—an essential prerequisite for a proper study of the liberation phenomenon itself.

Most bacterial exo-enzymes, including the α -amylase of *Bacillus subtilis* 6346 itself, appear to exist almost exclusively in the medium (Pollock, 1961*b*); in other words, they are liberated as soon as they are formed. This is obviously not an ideal situation for the purpose of studying enzyme liberation distinct from enzyme formation. Although, therefore, the *B. subtilis* penicillinase system may be atypical, it seemed to offer an exceptional opportunity for studying one aspect of the phenomenon of enzyme liberation. Our objective has, correspondingly, been limited: that of attempting to understand the mechanism by which the cell-bound fraction of *B. subtilis* penicillinase is liberated into the surrounding medium. In order to do this, the problem has been divided into three parts.

(1) Measurement and control of cell lysis of gross cell damage and assessment of its possible role in the liberation process (this article). (2) Attempts to identify the location of the cell-bound fraction of the enzyme (Kushner & Pollock, 1961). (3) Observations on the 'normal' enzyme liberation process itself (Pollock, 1961*a*). It has been possible to show, by means of the studies reported in this and the following two papers, that the liberation of penicillinase occurs with little or no cellular damage, under conditions dissociated from cell growth and multiplication, and involves detachment of the enzyme from the superficial layers of the cell by a process which probably has an enzymic basis.

METHODS

Organism. *Bacillus subtilis* NCTC 6346 was chosen for reasons outlined above and because it is also a good producer of α -amylase whose production and liberation are forming the subject of a parallel study.

Medium. The basal medium (CH) for all experiments contained 1% Casamino acids (Difco), 0.02 M-KH₂PO₄ adjusted to pH 7.2 with NaOH; 1.7×10^{-3} M-MgSO₄ + 0.1 ml. 'stock iron' solution (mixture of 0.156% ferrous ammonium sulphate + 0.168% citric acid per 100 ml.) + 0.1 ml. trace metals mixture (= 'oligodynamic solution' as used by Pollock & Kramer, 1958) per 100 ml.

Growth conditions. Unless otherwise stated, cultures were incubated by shaking aerobically at 35°. After inoculation the previous night with a few drops of a standard spore suspension (3×10^8 viable spores/ml.), cultures were first incubated without shaking for about 16 hr. and then transferred in the morning to a shaker at 35° in a conical flask containing not more than 1/5 its volume of medium, and grown under fully aerobic conditions during the whole of the experiment.

Enzyme induction. Penicillinase was usually induced by the addition of 0.06 μ g. (= 0.1 unit)/ml. benzylpenicillin (Glaxo 'Crystapen'). In some experiments, where larger amounts of penicillinase were required, 1 μ g. or 10 μ g./ml. of the non-metabolizable inducer, cephalosporin C, was used instead. α -Glucosidase was induced by

the addition of 0.25 mg. maltose (BDH 'Analar')/ml. Unless specifically stated otherwise, both enzymes were induced when the culture density reached the equivalent of 0.1 mg. bacterial dry wt./ml. and this point has been used as a standard zero reference time in all experiments—e.g. '3 hr. cells' means cells from a culture in CH medium 3 hr. after the point where an opacity equiv. 0.1 mg. dry wt./ml. was reached.

Enzyme assays. Samples (usually 5 ml.) were removed from the culture when required and cooled rapidly in ice. Two to three ml. were then spun at 20,000 *g* in the cold room for 10 min. and the supernatant fluid removed for assay of released enzyme. One drop of a 2 mg./ml. solution of egg-white lysozyme (Armour Labs.) was added to the remainder of the sample which was incubated at 35° until lysis was complete (2–5 min.) and then kept at 0° until assay of total enzyme. This procedure prevented any further production of enzyme and allowed full expression of α -glucosidase activity which in intact cells assayed at only about 40 % of the activity found after lysis or other methods (e.g. toluene treatment) of disrupting cell structure. Neither toluene nor lysozyme had any effect on penicillinase activity.

Penicillinase was assayed at 30° either manometrically on 1.0 ml. samples (Henry & Housewright, 1947) at pH 7.0, or iodometrically at pH 6.5 (Perret, 1954), the latter method being preferred under conditions of high buffer concentration or at pH values removed from neutrality. As used in this work, the manometric method gave approximately 25 % lower values, partly because of the CO₂ retention due to phosphate buffer in the medium. No correction was made for this, since it could be assumed that it was the same for all samples; comparisons were of course confined to results obtained by the same method, and conclusions were not in any way affected by the discrepancy. When dialysed samples of lysozyme-lysed, penicillin-induced cultures were assayed for penicillinase activity by both methods, the manometric technique still gave results 15 % lower than the iodometric method. The agreement is reasonable, and excludes the possibility of there being any significant penicillin-amidase activity present which might theoretically give misleadingly high results in the manometric assay technique for penicillinase (= penicillin β -lactamase). Penicillin-amidase is now known to be formed by certain bacterial species (Rolinson *et al.* 1960) though it has not been reported as occurring in the *Bacillus* genus. Its presence might possibly contribute to the release of CO₂ from penicillin and bicarbonate in the manometric assay for penicillinase, but would not interfere with the iodometric assay since the products of its action on benzylpenicillin (phenylacetic acid and 6-aminopenicillanic acid) do not react with iodine. If penicillin-amidase were present in significant quantity any discrepancy that might result between the iodometric and manometric assays of penicillinase would be in the opposite direction from that which was actually observed.

One ml. of 1 % (w/v) gelatin was originally included in the manometer cups for protection of the enzyme against possible loss or inactivation (see Pollock & Perret, 1951), but this was discontinued when it was discovered that *Bacillus subtilis* penicillinase is much more stable than the *B. cereus* enzyme and is not absorbed by glass to any significant extent (Kushner, 1960). All results are expressed in units of enzyme/ml. (as defined by Pollock & Torriani, 1953).

α -Glucosidase was assayed by measuring the absorption at 420 m μ (in a Unicam spectrophotometer) of the *p*-nitrophenol liberated by the enzyme after hydrolysis

of *p*-nitrophenol- α -D-glucoside, in comparison with standard concentrations of *p*-nitrophenol prepared by dissolving 0.1 g. in 500 ml. water and making suitable dilutions in 0.05 M-sodium phosphate buffer (pH 7.0) to 5.0 ml. lots of which was added 1 ml. of M-K₂CO₃. One ml. samples of enzyme (diluted, when necessary, with water) were mixed with 3.5 ml. of 0.05 M-sodium phosphate buffer (pH 7.0) in a 35° water bath and the reaction started by the addition of 0.5 ml. of a 1.0 mg./ml. solution of the substrate. The reaction was stopped by the addition of 1.0 ml. M-K₂CO₃ after sufficient colour had developed. Where necessary, the reaction mixture was clarified by centrifugation at 3000 rev./min. for a few minutes. Blanks, prepared by incubating the ingredients under similar conditions (1 ml. of water being substituted for the enzyme samples) were used for comparison in all experiments. It was shown that under these conditions the reaction was linear for at least 60 min. incubation and that the rate was proportional to the enzyme concentration, as long as no more than 80% of the substrate was hydrolysed. Activities are expressed as the quantity of *p*-nitrophenol formed/hr./ml. of original enzyme preparation. The *p*-nitrophenol- α -D-glucoside was prepared according to the method of Aizawa (1939), and gave a m.p. of 210°.

β -Galactosidase was assayed at 35° and pH 7.0 by the method of Lederberg (1950) with *o*-nitrophenol- β -D-galactoside as substrate, activities being expressed as the quantity of *o*-nitrophenol produced/hr./ml. original enzyme preparation.

Opacities of bacterial suspensions were measured in a Hilger Spekker absorptiometer with a neutral H 508 filter and expressed in terms of the equivalent bacterial dry weight as read off on a standard calibration curve prepared with suspensions of *Bacillus subtilis* of known dry weight values.

Total protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) against a bovine serum albumin standard, after precipitation with 5% (w/v) trichloroacetic acid (TCA) at 90° for 20 min. and subsequent washing of the precipitate twice with 5% TCA before dissolving in N-NaOH.

'260 m μ absorption' was measured directly in a Unicam spectrophotometer after making suitable dilutions in 10⁻² M-KH₂PO₄ (pH 7.0) solution.

RESULTS

Kinetics of release

Figure 1 shows the production and release of penicillinase from *Bacillus subtilis* 6346 growing in CH medium after induction with 0.06 μ g. benzylpenicillin/ml. at 0.1 mg. bacterial dry wt./ml. About 3 hr. after induction, formation of the enzyme practically ceased (quite unlike the situation with *B. cereus* where penicillinase synthesis continues apparently indefinitely), whereas cells went on growing and release of the enzyme persisted until, after overnight incubation, it had all been liberated into the medium. This means that, subsequent to a point 3 hr. after induction, liberation is spontaneously dissociated from production. Nearly all investigations on the liberation process were therefore confined to the first 2-3 hr. of this period. Cessation of penicillinase production at the '3 hr. point' was shown to be due to an induction failure, since on further addition of benzylpenicillin to '3 hr.' cells, production recommenced, and when an inducer, such as cephalosporin C, which is not detectably hydrolysed by the enzyme, was added at the normal induction time

(0 hr.) production persisted for as long as the organisms were growing. The liberation phase, however, could be shown to be not markedly influenced either by the absolute rate of penicillinase formation or the interval elapsed following addition of the inducer. For instance, in the experiment summarized in Table 1 there was perhaps a significantly lower proportion of penicillinase liberated at 4 and 5½ hr. by cells induced at 2½ hr. as compared with cells induced with the same concentration of penicillin at 0 hr.; but in a culture induced at 4 hr. the percentage release only 1½ hr. later was as high as that shown by cells induced 5½ hr. previously. Table 1 shows also that the proportion of extracellular penicillinase in cultures induced with cephalosporin C was not significantly different from that in penicillin-induced cultures where there might be only one-eighth as much total enzyme present.

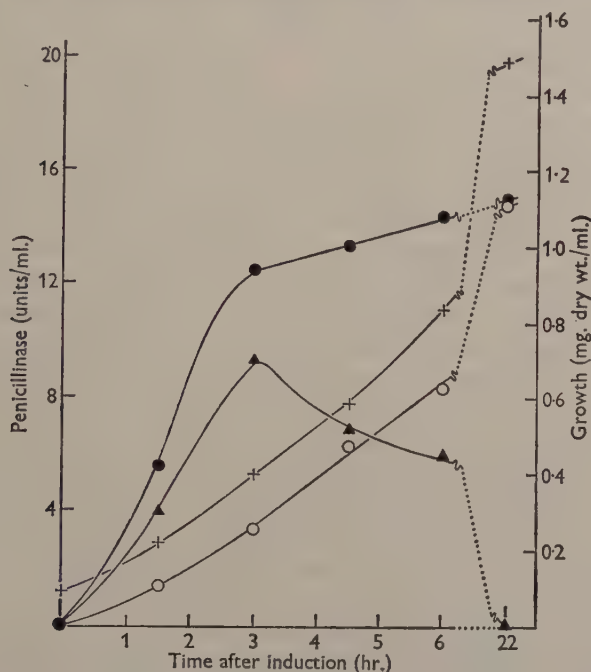


Fig. 1. Production and liberation of penicillin-induced penicillinase by cells of *Bacillus subtilis* 6346 growing in casein hydrolysate. Total penicillinase (—●—●—); penicillinase liberated from cells into medium (—○—○—); cell-bound penicillinase (—▲—▲—); growth opacity (—+—+—).

The release of the enzyme, therefore, seemed to be related to the physiological state of the culture rather than to the rate or stage of enzyme induction. Moreover, when relatively 'young' (2 hr.) cephalosporin C-induced cells were treated with 40 µg. chloramphenicol/ml. and incubated in the supernatant fluid from an 'old' (5½ hr.) uninduced culture (after readjusting to pH 7.0 with N-HCl and supplementing with a further addition of 1% (w/v) Casamino acids), they were found to liberate the same quantity of penicillinase into the medium as did the cells of a similarly induced and chloramphenicol-treated control culture incubated in fresh medium. It would appear, therefore, that release of the enzyme must depend upon changes in the cells themselves and not factors produced or liberated in the medium.

State of released enzyme. The penicillinase released during the 3–6 hr. period appeared to be entirely in a soluble state. The titre (3.08 units/ml.) of induced enzyme activity in the supernatant fluid of a sample from a 5½ hr. culture centrifuged for 30 min. at 105,000 g was not significantly different from that (2.94 units/ml.) in the supernatant fluid of an sample spun for only 15 min. at 5000 g. Removal of cells by an entirely different method (filtration through an 'Oxoid' membrane) left nearly the same activity (2.60 units/ml.) in the cell-free filtrate.

The control and measurement of cell lysis and gross cell damage

Cells of *Bacillus subtilis* are notoriously prone to undergo lysis (see Nomura *et al.* 1958; Nomura & Hosoda, 1956), the metabolic basis of which is not known. Gross lysis, involving obvious diminution in opacity of bacterial suspensions, was frequently observed during incubation (a) under conditions of suboptimal aeration, (b) with limiting nutrients, (c) after cold 'shock' at 0°, (d) in the presence of various growth inhibitors. It always led to an increase in the release of penicillinase. Less severe and less obvious 'interference' with cultures, insufficient to cause an obvious decrease in suspension opacity, was often found to be enough to allow leakage of enzymes otherwise found fixed to growing cells, and this, too, was usually associated with increased liberation of penicillinase. On *a priori* grounds, such enzyme release might therefore not unreasonably be regarded as an indication of cell damage: i.e. 'unphysiological'. Thus, it was first necessary to develop a method for measuring relatively small degrees of cell damage and to find a means of preventing it. The ideal tool would seem to be what we have called an intracellular 'marker', whose presence in the extracellular medium should indicate the extent of damage under all experimental conditions. To be satisfactory, such a 'marker' should have the following properties. (1) It should *normally* (i.e. under conditions of growth where there are no *a priori* grounds for believing that cell damage occurs) be entirely 'cell-bound' (i.e. associated with the deposit on centrifugation of a culture at a speed at which all the organisms are spun down). (2) It should be rapidly, and if possible completely, released into the medium by procedures known to cause cell lysis and/or disruption or disintegration of the cell 'envelope' (which, for the purposes of this work, is taken to mean the cell wall plus cytoplasmic membranes plus any other intimately associated structures or material). (3) It should be stable when present in the medium of a normally growing culture of the organism being investigated. (4) It should be capable of being easily, rapidly and accurately estimated.

The first marker tested, deoxyribonucleic acid, proved to be extremely rapidly broken down by cells in a growing culture and was therefore discarded. The second choice, a rather feeble, constitutive β -galactosidase, appeared during preliminary studies to be fairly satisfactory, although there was some loss (20–30 %) of activity during incubation with a growing culture over the 3 hr. period being studied. It was eventually discarded after the discovery that although this enzyme was almost completely released into the medium on cell disruption, less drastic damage to the cytoplasmic membrane (e.g. by incubation of cells with 4 % butanol) allowed only a relatively slow escape from the cell. It was therefore conjectured (and afterwards confirmed) that minor degrees of damage were being missed.

The marker finally chosen was α -glucosidase. This proved to be reasonably

satisfactory and its activity, in whole cultures and in the medium alone, was assayed in every experiment relating directly to the phenomenon of penicillinase liberation. The enzyme has the additional advantage of being inducible (by maltose). This meant that it was possible to adjust conditions such that its production by the cells was restricted to the period when they were forming penicillinase. Thus, unless there was a non-uniform and different distribution of the two enzymes amongst the cells of a heterogeneous population it could the more reasonably be assumed that any release of α -glucosidase after the 3 hr. point (when penicillinase production largely ceased) reflected the condition of the same cells as those which were liberating penicillinase.

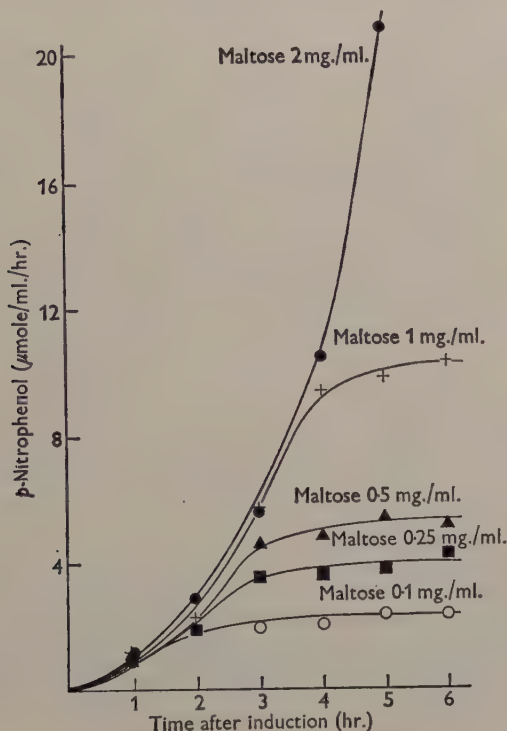


Fig. 2. The effect of different initial concentrations of maltose on the induction of α -glucosidase in a penicillin-induced culture of *Bacillus subtilis* 6346 growing in casein hydrolysate. The maltose was added at the same time as benzylpenicillin ($0.06 \mu\text{g./ml.}$), when bacterial suspension opacity reached the equivalent of $0.1 \text{ mg. dry wt./ml.}$ Initial maltose concentration of 2 mg./ml. (—●—●—); 1 mg./ml. (—+—+—); 0.5 mg./ml. (—▲—▲—); 0.25 mg./ml. (—■—■—); 0.1 mg./ml. (—○—○—).

Properties of the α -glucosidase system

Induction. The effect of different concentrations of maltose, added at the same time as the penicillin, on α -glucosidase formation, is illustrated in Fig. 2. It can be seen that at an initial concentration of $0.5 \text{ mg. maltose/ml.}$ and below there was little further production of the enzyme after 3 hr., due to exhaustion of the substrate. In order, therefore, to obtain reasonable α -glucosidase titres and at the same time to ensure that formation of the enzyme ceased at 3 hr. (i.e. at about the same time as penicillinase production faded out) an initial maltose concentration of

0.25 mg./ml. was chosen for all experiments. Addition of this concentration of maltose had no detectable effect either on cell growth or on penicillinase induction and liberation.

Assay. As mentioned above, the enzyme is partially cryptic, and lysis by lysozyme was used to allow full expression of activity. With a soluble preparation of the enzyme obtained from the supernatant fluid of a suspension of maltose-induced cells crushed at -30° in the Hughes's press, the pH value for optimum activity was found to be between 6.5 and 6.75 in 0.1 M-sodium phosphate buffers. The enzyme was stable during incubation for 2 hr. at 35° in 0.1 M-phosphate buffers from pH 6.0 to 8.2.

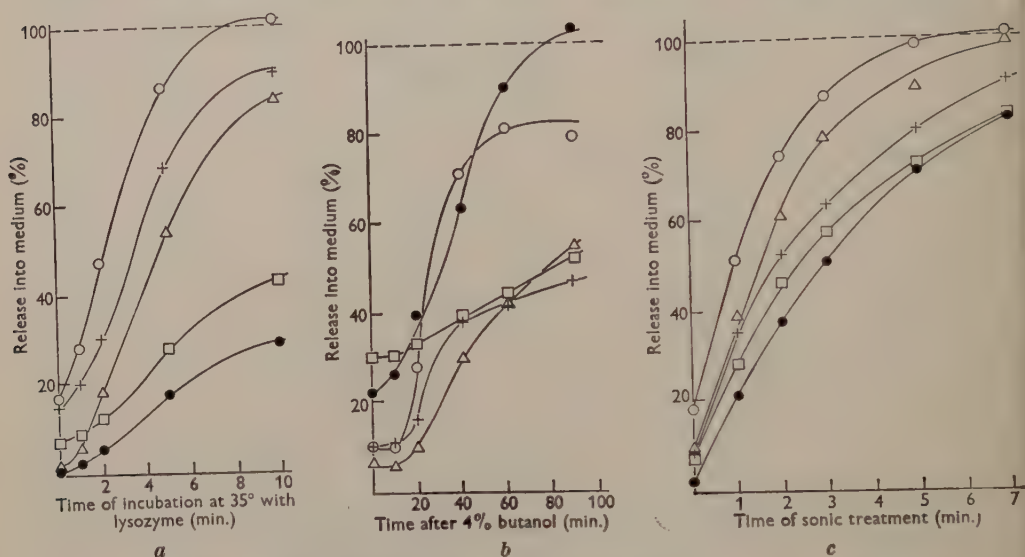


Fig. 3. Release of α -glucosidase ($\circ-\circ-$), β -galactosidase ($\triangle-\triangle-$), penicillinase ($\bullet-\bullet-$), total protein ($+ - + - +$) and material absorbing at $260\text{ m}\mu$ ($\square-\square-$) from cells of suspensions of *Bacillus subtilis* 6346, previously induced with maltose and benzylpenicillin, following treatment: (a) with $100\text{ }\mu\text{g}$. lysozyme/ml. + 0.05 M-MgSO_4 at 35° ; (b) with 4% butanol at 35° ; (c) by sonication at $0-10^{\circ}$. In all experiments, the first samples were taken immediately after beginning of treatment. (For further technical details, see text.)

Rate and extent of release from disrupted cells. α -Glucosidase was more rapidly released from cells, after disruption or damage by a variety of procedures, than any other cell constituent measured. Figures 3a, b and c show the release of α -glucosidase, β -galactosidase, penicillinase, total protein and $260\text{ m}\mu$ -absorbing substances from sedimentable material ($20,000\text{ g}$, 15 min.) after treatment of 3 hr. *Bacillus subtilis* cells (doubly induced with maltose and benzylpenicillin): (a) with lysozyme ($100\text{ }\mu\text{g./ml.} + 0.05\text{ M-MgSO}_4 + 0.01\text{ M-KH}_2\text{PO}_4$ buffer pH 7.0), after centrifugation and resuspension to $\frac{1}{2}$ vol. at 35° ; (b) with 4% butanol added directly to the culture at 35° ; (c) by sonication at 25 Kc/min. at $0-10^{\circ}$ (after centrifugation of the culture and resuspension in fresh medium at $13.0\text{ mg. dry wt./ml.}$). In all experiments samples were removed at the times indicated, the '0 min.' sample being taken immediately after the beginning of treatment, cooled to 0° in an iced bath and thereafter centrifuged at 2° as soon as possible.

Table 1. *Effect of time and type of induction on the release of penicillinase from cells of a Bacillus subtilis culture in casein hydrolysate*

Time of induction* (hr.)	Penicillinase activities (units/ml.) after following times											
	2½ hr.			3 hr.			4 hr.			5 hr.		
	Total	Sn† released	% released	Total	Sn	% released	Total	Sn	% released	Total	Sn	% released
Benzylpenicillin (0.06 µg./ml.)	11.4	2.4	21	.	.	.	14.2	4.2	30	.	.	.
	8.4	1.43	17	.	.	.

Cephalosporin C (10 µg./ml.)	50.5	9.6	19
	.	.	.	110	28.6	26	125	41	33	148	52	35

* Measured from point when optical density of culture reached the equivalent of 0.1 mg. dry bacterial wt./ml.

† Sn = supernatant fluid.

The '100 % release' value for comparison was obtained by assaying a measured sample of cells resuspended to the same cell density in buffer without Mg^{++} , after addition of 100 μg . lysozyme/ml. and incubation at 35° to obtain full lysis.

After crushing a 13.0 mg. dry wt./ml. suspension of cells in 0.01 M-sodium phosphate (pH 7.0) + 0.05 M- $MgSO_4$ in a Hughes's Press at -30°, 88 % of the enzyme was found to be released into the fraction unsedimented by centrifugation at 20,000 g at 2° for 15 min.

An analogous, dissociated release of up to 90 % of the α -glucosidase, with no more than 5 % release of penicillinase occurred when cells were treated at 0°, especially in the presence of salts (see Pollock, 1961a).

Stability in culture medium. An α -glucosidase preparation was obtained from the supernatant fluid of a 3 hr. maltose-induced culture of *Bacillus subtilis* lysed by subjection to 30 min. incubation at 35° without aeration. This was added at a dilution of 1/8 to a 3 hr. culture in CH medium, previously induced for penicillinase but not for α -glucosidase, growing at 35°, exactly under the conditions used for studying penicillinase release. The enzyme in the supernatant fluid was assayed at the beginning of the experiment and after 2½ hr. further incubation (see Table 2). The spontaneous release of α -glucosidase from cells of a maltose-induced sister culture, incubated under similar conditions, but without the addition of the enzyme to the medium, was followed in the same experiment.

The α -glucosidase was found to be inactivated by certain proteinases: trypsin (Armour Labs.); crystalline *Bacillus subtilis* N proteinase (Nagase and Co. Ltd.) and a concentrated supernatant fluid from older cultures of *B. subtilis* 6346 itself, known to have proteolytic activity. This proteolytic destruction was partially prevented by 10^{-4} M-diisopropylfluorophosphate (DFP) and almost completely (90 %) inhibited by 0.1 % heparin (Roche Products Ltd., 110 I.U./mg.). It seemed therefore important to know in this experiment whether the addition of heparin and other proteinase inhibitors had any effect both on the amount of α -glucosidase activity found in the supernatant fluids of cultures which were likely to be releasing the enzyme spontaneously, and on the stability of the enzyme added to cultures not forming it. None of the added substances had any significant effect on increase in bacterial suspension opacity. The results (Table 2) indicate in both experiments that, whereas soybean trypsin inhibitor (Worthington Biochem. Corporation) had no 'protective' effect, the activity in the supernatant fluid of cultures without either heparin or DFP was up to 10 % lower than in the others, therefore a very slight degree of inactivation may have occurred. Clearly, however, this was not enough to invalidate the use of the enzyme as a satisfactorily stable 'marker' in this study.

Results with α -glucosidase as 'marker'

'Damage-release'. By taking the release of preformed α -glucosidase into the medium as a criterion of cell damage, it was soon apparent that *Bacillus subtilis* cells were even more susceptible to harm than had previously been suspected. For instance, even removal of a culture from the shaker and allowing the flask to stand on the bench for a few minutes (such as might sometimes be necessary for sampling, etc.) could allow the subsequent release (after the culture was returned to fully aerated conditions) of up to 40 % of its α -glucosidase within 2 hr. without any significant decrease in optical density of the suspension. A similar degree of what

Table 2. *Stability of α -glucosidase in the culture medium of Bacillus subtilis growing in casein hydrolysate and the effect of certain proteinase inhibitors*

Culture	Additions (at 3 hr.)	α -Glucosidase activities (m μ mole <i>p</i> -nitrophenol formed/ml./hr.)			
		3 hr.		5½ hr.	
		Total	Sn*	Total	Sn
Induced with penicillin and maltose	Control (no additions)	2500	69	2660	86.5
	+ trypsin inhibitor 1 mg./ml.	.	.	.	86.5
	+ 0.1 % (w/v) heparin	.	.	.	110
	+ 3×10^{-4} M DFP	.	.	.	103
	+ Sat. soln. DFP	.	.	.	114
Induced with penicillin, but not maltose; α -glucosidase preparation added at 3 hr.	Control	353	255	.	285
	+ trypsin inhibitor, 1 mg./ml.	.	.	.	284
	+ 0.1 % heparin	.	.	.	304
	+ 3×10^{-4} M-DFP	.	.	.	297
	+ Sat. soln. DFP	.	.	.	316

For full details, see text.

Sn = supernatant fluid.

might be called 'damage-release' of α -glucosidase resulted from a single centrifugation of cells and resuspension in 0.01 M-sodium phosphate buffer, or even in the same medium.

More pronouncedly rough handling of the cells and longer periods of anaerobiosis led, as might be expected, to obvious lysis. But even the smaller degree of α -glucosidase leakage that resulted from minimal interference with standard conditions of culture was usually reflected (as long as it amounted to at least 20 % of the total) by a detectable lag in growth, although no lysis might be apparent, thus justifying the conclusion that escape of this enzyme is 'unphysiological'. Figure 4 shows the effect on α -glucosidase release of 20 min. semi-anaerobic treatment (removal from the shaking apparatus and left static at the same temperature) of a 3 hr. culture subsequently incubated with full aeration, in comparison with a control sister culture maintained under conditions of full aeration all the time. An even more striking example of cell-damage, caused by only 5 min. treatment of a 3 hr. culture at 0° (removal from 35° bath and incubation, with continued shaking in an ice bath), is illustrated in Fig. 5. This cold 'shock' led to the release of nearly 30 % of the total α -glucosidase during the first 30 min. of the ensuing incubation at 35°; and this was associated with (a) a parallel 'damage-release' of about the same proportion of penicillinase, over and above the normal penicillinase liberation, and (b) a 20 min. lag in growth, as measured by increase in optical density.

'Physiological' release. As long as proper care was taken to avoid mechanical and metabolic damage to the cells, the α -glucosidase released from cells of a growing *Bacillus subtilis* culture during the usual 0–5½ hr. period was found never to exceed 5 % of the total, whereas anything up to 50 % of the penicillinase was liberated during the same period. In a series of six similar experiments covering the 0–5½ hr. period a mean α -glucosidase release of 3.4 % was associated with a mean penicillinase release of 36 % of the total. At an earlier stage (0 to 3 hr. period) the figures were

1.2 and 20.5 %, respectively. Figure 6 illustrates a typical experiment. It was not possible to follow the subsequent release of the rest of the cell-bound penicillinase under conditions where damage-release could be excluded.

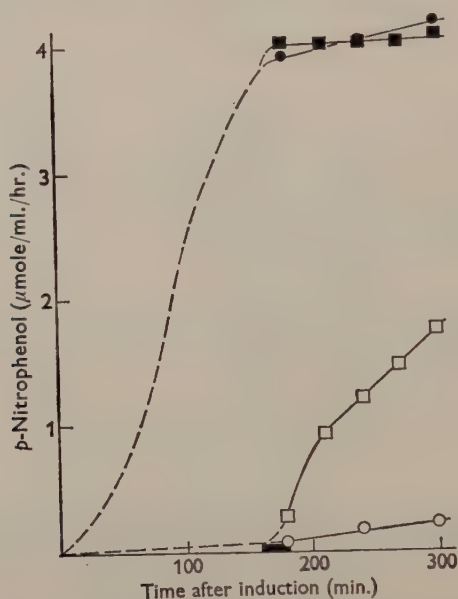


Fig. 4. The effect of transient semi-anaerobic treatment of a maltose-induced culture of *Bacillus subtilis* in casein hydrolysate on leakage of α -glucosidase from cells into the medium during subsequent aerobic incubation. Total α -glucosidase of control culture (—●—●—); total α -glucosidase of culture after 20 min. semi-anaerobic treatment (—■—■—); α -glucosidase released into medium from control culture (—○—○—); α -glucosidase released into medium from culture after 20 min. semi-anaerobic treatment (—□—□—); 20 min. period of semi-anaerobic treatment to 'test' culture (removal from shaking apparatus for 20 min.) indicated by ■■■.

DISCUSSION

The fact that cell damage in *Bacillus subtilis* leading to release of α -glucosidase, associated with increased liberation of penicillinase, could take place after apparently slight interference with the normal conditions used for growing and handling the organisms, emphasized the importance of careful control of all techniques. Apart from the dangers of transient deprivation of O_2 already mentioned, it was also found that a high proportion of substances, subsequently tested for their possible effect on penicillinase liberation, promoted release of α -glucosidase (see Pollock, 1961a). It seemed clear that any release of α -glucosidase above the irreducible minimum of up to 5 % observed even under 'normal' conditions, would have to be regarded as indicating presumptive cell damage (probably to the cytoplasmic membrane) and that any associated increase in penicillinase liberation must be considered unphysiological and therefore, according to our criterion, an artefact. Once the conditions which caused this α -glucosidase leakage were known, the greatest care was taken to avoid them (e.g. sampling was carried out without removing culture flasks from the shaker). This 'damage-release' appears to be a

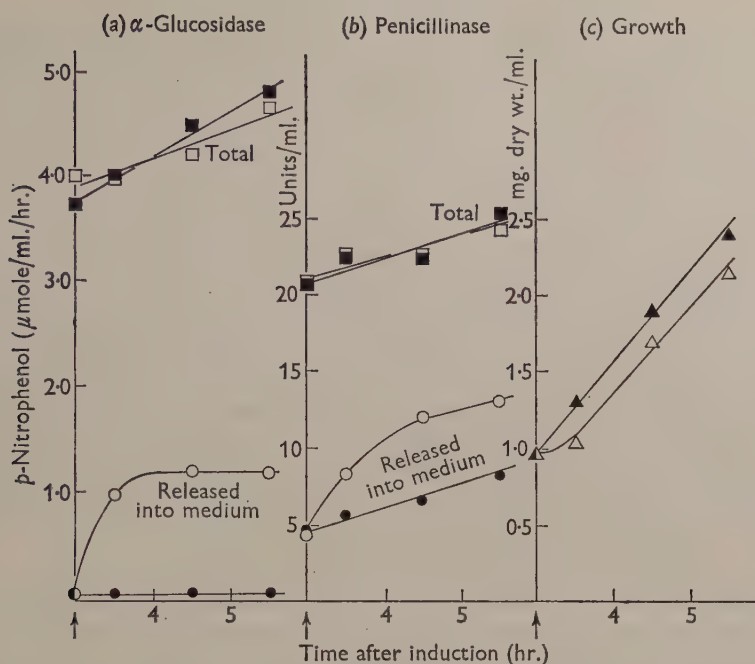


Fig. 5. Effect of transient treatment (5 min.) at 0° of a penicillin- and maltose-induced culture of *Bacillus subtilis* growing in casein hydrolysate at 35° , on leakage of α -glucosidase, and associated 'damage-release' of penicillinase from cells into the medium. Total enzyme (square symbols: \blacksquare , \square); enzyme in culture supernatant (circular symbols: \bullet , \circ); growth (triangular symbols: \blacktriangle , \triangle); control culture (filled-in symbols: \blacksquare , \bullet , \blacktriangle); culture treated 5 min. at 0° , at point indicated by arrow (open symbols: \square , \circ , \triangle). (a) α -glucosidase; (b) penicillinase; (c) growth (optical density).

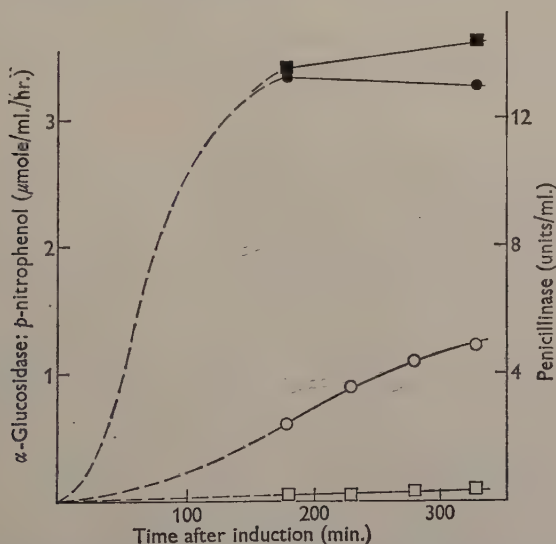


Fig. 6. Liberation of penicillinase and α -glucosidase (used as 'intracellular marker') from apparently undamaged cells of a penicillin- and maltose-induced culture of *Bacillus subtilis* growing in casein hydrolysate. Total penicillinase (— \bullet — \bullet —); penicillinase liberated into medium (--- \circ — \circ —); total α -glucosidase (— \blacksquare — \blacksquare —); α -glucosidase liberated into medium (--- \square — \square —).

secondary phenomenon, associated with autolytic processes which are initiated by cell damage and inhibited by the higher concentrations (0.05 M) of Mg^{++} (see Pollock, 1961*a*), which are known to assist in maintaining the integrity of the cytoplasmic membrane and other cell structures (Weibull, 1956). It now seems probable that the mechanism of the increased penicillinase release which occurs in association with the conditions that lead to α -glucosidase release is quite different from the normal liberation which occurs in cultures of apparently undamaged organisms.

It can, of course, be argued that, had an intracellular 'marker' more sensitive than α -glucosidase been available, some evidence of cell damage might have been disclosed during the period when what has been referred to as 'physiological' liberation of penicillinase was occurring. There is no formally satisfactory answer to this objection. A great deal must depend upon what is accepted as a definition of cell 'damage'. Clearly, organisms releasing an enzyme into the medium at a high rate must in some way be different from organisms of the same culture which liberate the same enzyme much more slowly. In this connexion, it is unfortunate that it proved to be impossible to do accurate viable counts on the *Bacillus subtilis* strain used in this study, since a comparison of these with total counts might have provided a completely independent assessment of what many people would readily accept as cell 'damage'. The ratios of viable counts to bacterial suspension opacity, however, even in samples from the same culture, were extremely variable and usually very low; no confidence whatever could be placed in their significance. Under the circumstances, however, it seems not unreasonable to assume that a good indication of cell damage is provided by the measurement of α -glucosidase release. It is therefore fair to conclude that a high proportion (up to 40%) of the cell-bound penicillinase of *B. subtilis* is liberated from the organisms by a process not involving lysis of cells or gross interference with their physiological functions.

I would like to thank Miss Joan Fleming for patient and expert technical assistance in this work, and Dr E. P. Abraham for gifts of cephalosporin C.

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The Location of Cell-bound Penicillinase in *Bacillus subtilis*

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SUMMARY

Before penicillinase is released from a penicillin-induced culture of *Bacillus subtilis* it accumulates and up to 85 % is found to be bound to the cells. This cell-bound penicillinase is not free in the cytoplasm since it cannot be released in a soluble state simply by rupture of the cell envelope. It appears to be attached to the cell in a fairly superficial location since up to 40 % is accessible to antiserum and up to 98 % can be released, by trypsin in the presence of hypertonic sucrose, from apparently intact cells.

INTRODUCTION

Most people who have considered the problem of exoenzyme liberation from living cells appear either explicitly or implicitly to suppose that the process must involve the passage of the enzyme from 'inside' the cell (where it is assumed to be formed) across the cell membrane to the extracellular environment. With bacteria, there is no direct evidence that this is so. One essential step in the study of the liberation process is clearly to determine the location of the enzyme whilst it is still associated with the cell. Until that is known, the experimental approach to the problem of its release may be disorientated. In addition, exact knowledge of the location of the cell-bound enzyme might help to solve the question of the site of its formation, a problem which is only just becoming susceptible to experimental attack. With most bacterial extracellular enzyme systems the proportion of activity found fixed to the cells is so low as to be barely detectable (see Pollock, 1961*a*). The penicillinase of *Bacillus subtilis*, however, is a striking exception to the rule, and for this reason is particularly susceptible to analysis in relation to the problem of enzyme liberation.

METHODS

The organism, medium and general techniques used and definitions adopted were identical with those described in detail in the preceding paper (Pollock, 1961*b*). Standard 'zero' reference time (0 hr.) from which all time periods were measured, was taken to be the point when the concentration of growing cultures reached the equivalent of 0.1 mg. bacterial dry wt./ml. Unless stated otherwise, this also was the time when cells were induced to form penicillinase and α -glucosidase.

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RESULTS

Role of the cell wall

Unlike the situation with *Bacillus cereus* (Sheinin, 1959), no evidence was obtained that a significant proportion of the cell-bound penicillinase of *B. subtilis* was attached to the cell by adsorption to the cell wall. Preparations of isolated cell walls (Cummins & Harris, 1956), Mickle-disrupted cells and intact cells from an uninduced 3 hr. culture of *B. subtilis* strain 6346 grown in casein hydrolysate (CH) medium, resuspended at equiv. 2 mg. dry wt./ml. in a concentrated supernatant fluid containing 100 units penicillinase/ml. from a penicillin-induced culture, in the presence of 0.01 M-KH₂PO₄ (pH 7.0) at 35° for 1 hr., were not found to have caused more than a 15% decrease in the penicillinase activity of the supernatant fluid after centrifugation, when compared with the total activity before centrifugation (Miss E. Janczura, private communication). In another experiment, three 4.0 ml. samples of a 3 hr. uninduced culture in casein hydrolysate medium (CH) were adjusted to pH 6.0, 7.0 and 8.0, respectively, and shaken at 35° after addition of 1 ml. of a concentrated supernatant fluid from a penicillin-induced culture, giving a final concentration of 50 units/penicillinase/ml. After 2½ hr., removal of the cells by centrifugation for 20 min. at 20,000 g did not result in any detectable decrease in penicillinase activity. This observation that *B. subtilis* penicillinase, unlike the *B. cereus* enzyme, was neither adsorbed to intact cells nor to isolated cell walls, may be related to the fact that, again in contrast to *B. cereus* penicillinase, it is not appreciably adsorbed to powdered glass (Kogut, Pollock & Tridgell, 1956; Kushner, 1960).

Effect of repeated cell washing

A 3 hr. penicillin-induced culture was centrifuged in the cold-room at 2° and the organisms resuspended to equiv. 2 mg. bacterial dry wt./ml. in ice-cold 0.02 M-KH₂PO₄ (pH 7.0). After removing a sample for assay (27.6 units penicillinase/ml.) the suspension was re-centrifuged, and the organisms washed four times successively in 10 ml. lots of iced buffer, being finally resuspended in the same volume of buffer and reassayed (28.2 units penicillinase/ml.). It is obvious that the enzyme was not removed by repeated washing.

Effect of cell disruption

Hughes's press. A suspension of standard 3 hr. penicillin-induced organisms at a concentration equiv. 20 mg. bacterial dry wt./ml. in iced 0.01 M-KH₂PO₄ buffer, (pH 7.0) + 0.05 M-MgSO₄ was crushed in a Hughes's press at -30°. When the crushed preparation was thawed and homogenized to obtain an evenly dispersed suspension, 43% of the total penicillinase activity was found in the supernatant fluid after centrifugation for 10 min. at 20,000 g.

Lysozyme treatment. 'Spheroplasts' (see McQuillen, 1960), prepared by incubating organisms with 100 µg. lysozyme/ml. at 35° in the presence of 0.3 M-sucrose or 20% (w/v) polyethylene glycol appeared, microscopically, to be stable, but were found to leak the normally cell-bound α-glucosidase (induced in the cells as an intracellular 'marker'; see Pollock, 1961 b) into the medium and released a variable proportion of penicillinase. The addition of high concentrations of Mg⁺⁺ (0.05 M-MgSO₄) decreased the rate of enzyme release during lysozyme treatment, and the

Table 1. *Liberation of penicillinase and α -glucosidase from disrupted spheroplasts of penicillin—and maltose-induced Bacillus subtilis 6346*

Treatment	Description	Penicillinase		α -Glucosidase	
		Units/ml.	% released to medium after treatment	μ mole <i>p</i> -nitrophenol ml./hr.	% released to medium after treatment
Resuspended organisms from 3 hr. culture	Whole organisms	16.80	.	5.70	.
Samples of whole organisms treated at 35° with 100 μ g. lysozyme/ml. + 0.3 M-sucrose for (a) 2 min., (b) 5 min., followed by centrifugation and resuspension in 0.05 M-MgSO ₄	Spheroplasts, prepared by lysozyme treatment for: (a) 2 min. (b) 5 min.	17.12 14.32	(-2) 14	2.42 1.95	57.5 65
'Spheroplasts', allowed to lyse in 0.05 M-MgSO ₄ , centrifuged and supernatant fluid retained	Supernatant fluid from disrupted spheroplasts, previously prepared by lysozyme treatment for: (a) 2 min. (b) 5 min.	0.34 0.50	2 3.5	2.24 1.92	93 96.5

For full experimental details see text.

spherical form was only slowly attained, although resuspension in dilute buffer solution (0.01 M-KH₂PO₄, pH 7.0, + 0.05 M-MgSO₄) without sucrose caused immediate lysis. This stabilizing effect appeared to be mainly on the cell membranes which disintegrated only very slowly as long as the Mg⁺⁺ concentration was kept high (as found with *Bacillus megaterium* by Weibull, 1956). The use of Mg⁺⁺ thus allowed the preparation of fairly stable 'membranes' (i.e. resuspended, lysed spheroplasts) which retained most of the penicillinase activity of the intact organisms, although nearly all the α -glucosidase had escaped into the medium during the lysozyme treatment and subsequent lysis in hypotonic buffer solution. Table 1 summarizes the results of an experiment which demonstrated this. A 3 hr. culture (90 ml.) induced with maltose and penicillin was centrifuged and the organisms resuspended at 35° in 45 ml. 0.01 M-KH₂PO₄ (pH 7.0) + 0.05 M-MgSO₄ and 0.3 M-sucrose. Lysozyme (100 μ g./ml.) was then added and 18 ml. samples removed after incubation for 2 min. and 5 min., cooled as rapidly as possible and centrifuged at 2° for 10 min. at 20,000 *g*. The deposits were resuspended to the same volume in iced buffer + 0.05 M-MgSO₄ and homogenized rapidly (2 min.) in a Potter-Elvehjem homogenizer, the temperature being kept as low as possible. Samples of these 'disrupted spheroplasts' were centrifuged for 10 min. at 20,000 *g* and the penicillinase and α -glucosidase activities remaining in the supernatant fluid compared with total activities before centrifugation and with the activities of a sample of the original cell suspension treated with lysozyme in the absence of sucrose or Mg⁺⁺. The results showed that (a) practically all the α -glucosidase was liberated when a lysozyme-treated preparation

of cells was lysed by resuspension in hypotonic medium, and that more than 50% escaped even before the sucrose was removed, whereas (b) barely significant amounts of penicillinase were released after only 2 min. lysozyme treatment and very little even after 5 min. As shown previously (Pollock, 1961 *b*), longer incubation resulted in further penicillinase liberation by processes which were considered to be secondary autolysis.

Table 2. *Liberation of penicillinase from disrupted 'spheroplasts' of Bacillus subtilis 6346*

	Total penicillinase (units/ml.)	Penicillinase released after incubation at 35° (units/ml.)	% released
Expt. 1: 90 min. incubation			
Control	13.6	7.8	58
+ MgSO ₄ 0.05 M	13.6	2.8	21
+ <i>p</i> -Chloromercuribenzoate 2.5×10^{-3} M	12.8	5.4	42
+ heparin 1 mg./ml.	14.4	8.0	56
+ diisopropylfluorophosphate 2×10^{-3} M	14.4	8.8	60
Expt. 2: 0.05 M-MgSO ₄ added to all tubes; incubation for 10 min.			
Control	17.8	4.4	25
+ trypsin, 1 mg./ml.	19.0	17.6	93
Expt. 3: 0.05 M-MgSO ₄ added to all tubes; incubation for 10 min.			
Control	32.4	7.1	22
+ trypsin, 1 mg./ml.	31.0	17.5	57
+ ribonuclease 1 mg./ml.	33.2	7.5	23
+ butanol 1 %	32.4	7.8	24
+ sodium deoxycholate 1 %	32.4	28.3	88

For full experimental details, see text.

This autolytic-type of penicillinase release from 'disrupted spheroplasts' (prepared as described above) was studied further in an experiment summarized in Table 2 where the effects of various reagents on the process were investigated. The following points may be seen. (a) Trypsin, at 1 mg./ml., rapidly liberated a large (though rather variable) proportion of the penicillinase even in the presence of 0.05 M-Mg⁺⁺. Sodium deoxycholate (10 mg./ml.) had a similar effect. (b) The 'autolytic' release was inhibited by Mg⁺⁺ (0.05 M) and by *p*-chloromercuribenzoate (2.5×10^{-3} M) but not by diisopropylfluorophosphate (DFP) or heparin. The question of how closely related this 'autolytic' liberation phenomenon may be to normal (physiological) release of enzyme in growing cultures is considered later.

Combined effect of trypsin and hypertonic sucrose

Trypsin alone (at least up to 1 mg./ml.) had no detectable action on growth or enzyme liberation from cultures of *Bacillus subtilis* 6346 in CH medium, and little effect on release of penicillinase or α -glucosidase from cells of a 3 hr. penicillin- and maltose-induced culture resuspended at 35° in phosphate buffer. But (see Table 2) it rapidly liberated the enzyme from a 'membrane'/preparation (disrupted 'spheroplasts'). Sucrose alone (0.9 M) completely inhibited growth and caused a variable degree of cell damage in similarly prepared suspensions of induced cells (as indicated

by α -glucosidase release into the medium) but had no effect on penicillinase liberation, at least during incubation for 30 min. at 35°. Treatment with trypsin and sucrose together, however, led to considerable release of cell-bound penicillinase within about 20 min. The extent of this effect, however, varied from day to day, maximal penicillinase release in 30 min. varying between the extremes of 45 and 98 % in different experiments. α -Glucosidase release also varied, but not in a fashion apparently correlated with the extent of penicillinase release. In the most striking experiments, a 98 % release of penicillinase was associated with only a 5 % release of α -glucosidase. Other typical experiments are shown in Table 3 and Fig. 1.

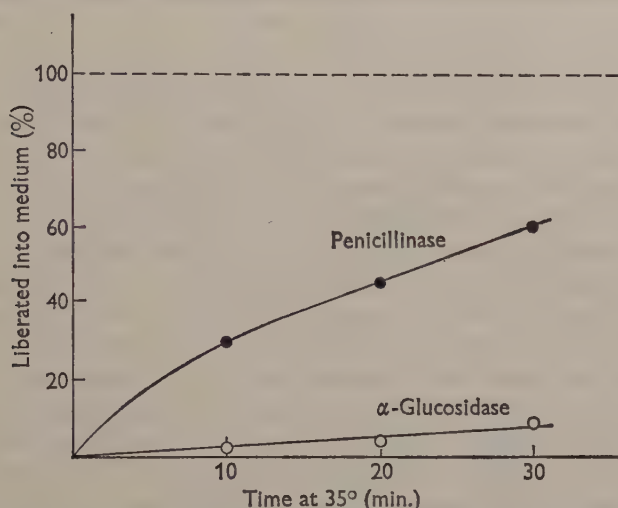


Fig. 1. The release of α -glucosidase (—○—○—) and penicillinase (—●—●—) from maltose- and penicillin-induced cells of *Bacillus subtilis* 6346 resuspended in 0.01M-sodium phosphate (pH 8.0) at 35° in the presence of 0.9M-sucrose + trypsin 1 mg./ml.

Table 3. Release of penicillinase from *Bacillus subtilis* 6343 by treatment with sucrose and trypsin for 30 min. at 35°

	Penicillinase units/ml.		Amount released into medium (%)
	Total	In supernatant fluid	
Control	19.8	1.8	9.1
+sucrose 0.9M	18.5	1.2	6.5
+trypsin, 1 mg./ml.	19.6	8.4	17.3
+0.9M-sucrose and trypsin 1 mg./ml.	18.9	13.1	69.5

For full experimental details, see text.

The full procedure for the above experiment was as follows. A 3 hr. culture of *Bacillus subtilis* 6346 induced with penicillin and with maltose was centrifuged, the organisms resuspended in different mixtures in 0.01M-KH₂PO₄ (pH 8.0) + 0.05M-MgSO₄, with or without 0.9M-sucrose and with or without trypsin 1 mg./ml. and all mixtures incubated aerobically at 35°. Samples were cooled rapidly, a few ml.

retained for total penicillinase assay and the remainder centrifuged at 20,000 *g* for 10 min. in the cold. The supernatant fluids were assayed for penicillinase activity, and the deposits resuspended to volume in a buffer mixture containing 0.02M-KH₂PO₄ (pH 7.0) and 0.1 % heparin (to inhibit the further action of trypsin on α -glucosidase as it is released) and 100 μ g. lysozyme/ml., incubated at 35° until lysis was complete and then assayed for α -glucosidase activity as soon as possible. The proportion of α -glucosidase released was calculated from the difference between the activities of the lysed deposits and those of the sample of original cell suspension lysed directly with lysozyme under the same conditions but with Mg⁺⁺, sucrose or trypsin. Whatever degree of penicillinase release was achieved, it was usually maximal after 20 min. incubation (although this particular feature does not happen to be well illustrated by the example shown in Fig. 1). This indicated that the inconstant results were due to variation either in the proportion of cells completely insensitive to the action of trypsin and sucrose or in the extent to which the cell-bound penicillinase had reached a location on the cells accessible to trypsin.

Trypsin had no effect on penicillinase, but rapidly inactivated the α -glucosidase as soon as it was liberated from the cell, though not before. The problem of assaying cell-bound α -glucosidase in the presence of trypsin was complicated by the need to lyse the cells with lysozyme in order to allow full expression of activity, after which the enzyme was rapidly destroyed by the trypsin. It had, however, been found that the inactivation of α -glucosidase by trypsin could be almost completely prevented by 0.1 % heparin (see Pollock, 1961*b*) which was therefore added as soon as samples were taken. In this way, it could be shown that as long as α -glucosidase remained within the cells, it was almost completely protected from the proteolytic action of trypsin. This suggested that the trypsin had not actually penetrated the cell membrane during the time it was releasing the penicillinase and must therefore be acting relatively superficially. At concentrations of sucrose below 0.9M (e.g. at 0.3M) the extent of penicillinase release by trypsin was relatively slight. It therefore seemed possible that liberation by trypsin was dependent on some degree of plasmolysis, perhaps involving separation of cell membrane and cell wall associated with surface damage, which allowed access of trypsin to certain structures (presumably fully exposed to the action of trypsin in the preparation of disrupted 'spheroplasts' described above) which were vital for the retention of penicillinase.

When 0.9M-sucrose was replaced by 4 % (w/v) polyethylene glycol (mean M.W. 400) which should be osmotically equivalent, no release of penicillinase occurred in the presence of trypsin. It is not, however, known whether polyethylene glycol can penetrate the cell wall. If this does not occur and the cell wall is not completely rigid, the cell may simply shrink as a whole and no plasmolysis involving separation of membrane and cell wall would take place. Whatever the nature of the bonds responsible for this binding of penicillinase, it seems likely that the trypsin could not have penetrated the barrier of the cytoplasmic membrane and that the bulk of the penicillinase, therefore, is probably bound on the outer surface of the membrane, or even more superficially.

No direct evidence, however, could be obtained on the mechanism of action of sucrose; neither observation by phase contrast nor electron microscope studies revealed any convincing difference between cells treated and untreated with 0.9M-sucrose, with or without trypsin. Amongst other enzymes tested because of their

ability to attack substrates which possibly form part of the superficial cell structure, the effect of trypsin was shared only by another proteolytic enzyme (*Bacillus subtilis* proteinase itself; Nagase and Co. Ltd., Itachibori-minamidori 1-chome, Osaka, Japan). Bovine pancreatic ribonuclease (Armour Pharmaceutical Company Ltd., Eastbourne), *Clostridium welchii* lecithinase (kindly given by Dr Marjorie Macfarlane of the Lister Institute of Preventive Medicine, Chelsea Bridge Road, S.W. 1), a pancreatic lipase preparation (Mann Research Laboratory, 136, Liberty Street, New York 6, N.Y.) and a microbial lipase preparation (Mann Research Laboratory, 136, Liberty Street, New York 6, N.Y.), all at a concentration of 1 mg./ml., tested in the presence of 0.9 M-sucrose, were without effect. The action of lysozyme has already been mentioned and discussed.

Experiments with anti-penicillinase serum

Specific neutralization by anti-enzyme serum has been used on several occasions as evidence for the localization of cell-bound enzyme on the cell surface (Sevag, Newcomb & Mill, 1954; Pollock, 1956*a*). The value of the method depends upon the assumption that rabbit γ -globulin cannot penetrate to 'within the cell' (i.e. pass through the cytoplasmic membrane), for which there is some experimental evidence in yeast (Krebs & Wright, 1951; Pasternak, Sevag & Miller, 1954). The extent to which it may or may not be able to pass through the cell wall is not known. Obviously, therefore, only positive enzyme neutralization tests can be satisfactorily interpreted. A negative result might mean that the enzyme was within the cytoplasmic membrane, or outside the membrane but protected by the cell wall from combination with antibody.

An antiserum against *Bacillus subtilis* 749 exopenicillinase prepared in rabbits was found to give 78% neutralization of the heterologous exopenicillinase from *B. subtilis* 6346 (Kushner, 1960). Cells from induced cultures of *B. subtilis* 6346 were centrifuged at times between 3 and 6 hr. after induction, resuspended to $\frac{1}{3}$ the original volume in 0.01 M-KH₂PO₄ (pH 7.0) containing 8×10^{-4} M-8-hydroxyquinoline to prevent further enzyme synthesis. Two lots of 0.5 ml. of this suspension were put into each of two Warburg flasks, one containing 0.2 ml. water and the other 0.2 ml. of anti-749 penicillinase serum (equivalent to approximately twice the quantity necessary for maximal neutralization of the enzyme). The flasks were left at room temperature for 20 min. Then 1.0 ml. of a mixture containing 1% (w/v) gelatin + 8×10^{-4} M-8-hydroxyquinoline, bicarbonate and penicillin was added as before, and the penicillinase activities measured. The results, giving '% enzyme in combination with antiserum' are calculated from the % difference in enzyme assay with and without antiserum, after allowing for the 22% residual activity of the enzyme + antibody complex consistently demonstrable, even in the presence of a gross antibody excess. It was found that the addition of an equivalent quantity of lysed or intact organisms from an uninduced culture had no significant effect on the neutralization of soluble enzyme by antisera, and that therefore the presence of cells was unlikely *per se* to affect the reaction between enzyme and antibody. Control experiments showed that negligible quantities of enzyme were liberated during the period required for allowing the antiserum to neutralize the enzyme and subsequent assay.

When the % cell-bound penicillinase combining with antiserum was measured in the period 3–5 hr. after induction, it was found, despite somewhat variable results,

that there was usually 30–40 % demonstrable at 3 hr. (after which there is little further production of enzyme in penicillin-induced cultures; Pollock, 1961*b*), but that after a further 2 hr. growth the proportion had fallen to 0–15 % (Fig. 2). By 5–7 hr., up to 60 % of the total enzyme formed had been liberated. The fact that the proportion of cell-bound enzyme which combined with antiserum tended to fall from 3 hr. onward suggested that it was this moiety which was first released. Calculations in individual experiments showed that the amount of enzyme released was sufficient to account for the decrease in antibody-combinable enzyme. These results also show, however, that enzyme release can take place from cells in which little or no neutralizable enzyme is detectable; this suggests that though enzyme molecules in the antibody-combinable state may be preferentially released, such a state is not necessarily a preliminary one to release from the cell.

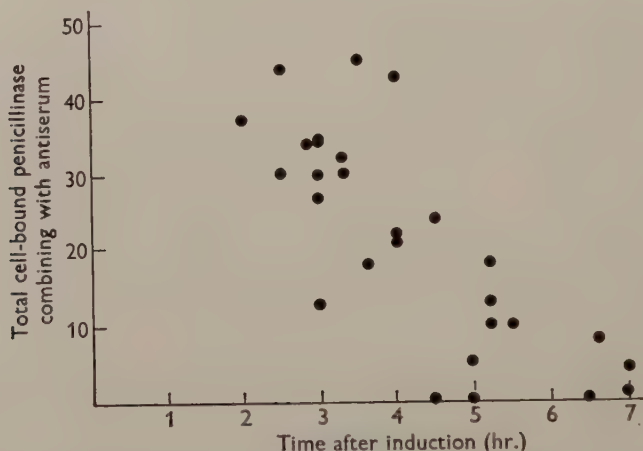


Fig. 2. A scatter diagram summarizing all results obtained in a series of experiments showing the decrease in the proportion of cell-bound *Bacillus subtilis* penicillinase combinable with anti-exopenicillinase serum, during the 2–7 hr. period following the point when the opacity of a culture growing in casein hydrolysate reached the equivalent of 0.1 mg. bacterial dry wt./ml. (being also the time when enzyme formation was induced with penicillin).

The proportion of antiserum-combinable cell-bound enzyme at 3 hr. after induction was not altered by conditions which increased the total amount of enzyme formed. In one experiment, where the culture had been induced with cephalosporin C (10 μ g./ml.) and the total quantity of penicillinase produced was nearly five times as great as that of a sister culture induced with penicillin, the above proportions were not significantly different (30 and 32 %, respectively). It should be recalled that increasing the total enzyme formed, by induction with cephalosporin C, did not influence the extent to which it was liberated into the medium (Pollock, 1961*b*). After overnight incubation of the culture, all the enzyme was liberated into the medium and was then found to be neutralized by antiserum to maximal extent, as with enzyme liberated in earlier stages of growth. It is thus clear that the cell-bound penicillinase has no immunologically distinct fraction similar to the ' γ -penicillinase' possessed by *Bacillus cereus* (Pollock, 1956*b*).

When 3 hr. cells of *Bacillus subtilis* 6346 were disrupted with lysozyme, not all the penicillinase was thereby immediately made accessible to antiserum, though the proportion rose from 40 to 60 % (means of 6 experiments). A similar increase was observed when 3 hr. cells were crushed in a Hughes's press. When 5 hr.-cells, which had already released most of their antiserum-accessible enzyme to the medium, were treated with lysozyme, the proportion increased from 11.5 to 53 % (means of 2 experiments). When lysed or crushed organisms were incubated overnight at 35°, the proportion further increased to 70–90 %, possibly through an autolytic destruction of bonds which prevented access of antibody to the relevant part of the enzyme molecule.

DISCUSSION

These results show that the cell-bound fraction of *Bacillus subtilis* 6346 penicillinase is largely, if not entirely, bound to insoluble cell structures, sedimentable by 15 min. centrifugation at 20,000 g. This fact, and the visible appearance of the cell debris following treatment with lysozyme in the presence of high concentrations of Mg^{++} , suggest that the enzyme is probably bound to structures forming the cell envelope. At least 30 % of the cell-bound fraction is fixed superficially to the cell, being accessible to combination with antiserum. The results with antiserum also suggest that it is this superficially-bound fraction of enzyme which is selectively released during the 3–6 hr. period during which the liberation process has been studied.

The action of trypsin in liberating a high proportion of cell-bound penicillinase after treatment of the cells with hypertonic sucrose, but apparently without penetrating the cytoplasmic membrane, suggests that the penicillinase was probably released from structures outside the main permeability barrier of the cell. This effect appears to be analogous to the action of trypsin in promoting the release of the 'M' protein from streptococci (Elliott, 1945). With streptococci, no preconditioning with sucrose or other adjuvant is necessary. The M protein appears to be bound directly or indirectly to cell wall material since it is not normally liberated into the medium in significant amounts except in old cultures, whereas it is not found attached to protoplasts which, nevertheless, appear still capable of forming it (Freimer, Krause & McCarty, 1959). A proportion of this protein must likewise be superficially located since it can be shown to react with antiserum in undamaged cells. On the whole it would appear that *B. subtilis* 6346 penicillinase may be fixed to the cells in a similar manner, but perhaps rather more deeply buried than is the M protein.

The results as a whole are consistent with the hypothesis that cell-bound penicillinase is attached to the outside of the cytoplasmic membrane, and may actually be formed at its surface. It is possible that at least a portion may be bound in, or closely associated with, the cell wall. Nor is it excluded that part might be in a soluble state, sandwiched between the cell membrane and an impermeable or poorly permeable cell wall (e.g. in the so-called 'periplasm' postulated by Mitchell, 1961). But it is difficult to understand, if this be so, how most of the α -glucosidase is released from the cells under conditions (e.g. incubation at 0°; Pollock, 1961*c*; or after brief lysozyme treatment with high Mg^{++} concentrations) which do not allow escape of penicillinase. The only evidence possibly in favour of the enzyme being in a 'periplasm' is the rapid release of over 40 % of the penicillinase after crushing in a Hughes's press. However, in order to obtain evenly dispersed suspensions of

disrupted cell material by this technique (without which, of course, no comparison of particle-bound and soluble enzyme is valid) it is necessary to thaw out the frozen crushed suspension for several minutes and homogenize it thoroughly before centrifugation. It is possible that this may be time enough for secondary autolytic processes to cause a considerable amount of release of penicillinase from cell fragments. In any case, it seems at the moment reasonable to approach the problem of 'normal' release of *Bacillus subtilis* 6346 penicillinase on the basis of a working hypothesis that it may involve the detachment of the enzyme from structures on the surface of, or outside, the main permeability barrier of the cell, rather than passage through the barrier itself.

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The Mechanism of Liberation of Penicillinase from *Bacillus subtilis*

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SUMMARY

The effects of various factors on the release of penicillinase from apparently undamaged cells of a growing culture of *Bacillus subtilis* were investigated. The enzyme was not eluted from the cells by treating them with high concentrations of salt. Its liberation did not take place at all at 0°, and was nearly completely inhibited at pH values below 6·0, whereas chloramphenicol, at concentrations sufficient to cause complete cessation of growth, caused only partial inhibition of enzyme release. The penicillinase-releasing action of extracts containing heat-labile 'autolytic' factors from older cells of the same organism could not be dissociated from their damaging effect on the cell, as indicated by concomitant release of the normally intracellular α -glucosidase. It is concluded that normal penicillinase liberation is controlled by enzymic reactions, as yet unidentified, involving detachment of the enzyme from structures superficially located in the cell envelope.

INTRODUCTION

In the first two papers of this series some evidence was presented to suggest that the liberation of penicillinase from a growing culture of *Bacillus subtilis* 6346 may involve a release of the enzyme from the superficial layers of apparently intact cells. The present paper records the results of a direct investigation of the liberation process itself, with a view to characterizing it and obtaining further information on its nature. Minor degrees of cell damage, insufficient to cause measurable lysis, were detected by following the leakage into the medium of an 'intracellular' α -glucosidase whose synthesis was induced in the cells, during the period when penicillinase was being formed, by the addition of a small quantity of maltose (Pollock, 1961*b*).

'Normal' or 'physiological' penicillinase liberation is defined here as the penicillinase release which occurs in untreated cultures in the absence of cell damage sufficient to cause leakage of α -glucosidase greater than the irreducible minimum of up to 5% that was always liable to take place. On this criterion, the conditions under investigation in many experiments caused significant cell damage, and in such cases it could only be concluded that the associated increase in penicillinase liberation, which nearly always occurred, was 'unphysiological'. Unfortunately this took place all too frequently, and in most of the other experiments the factors being studied had no detectable effect. Our conclusions have therefore been limited, extending only to an indication that 'normal' penicillinase liberation is not a matter only of physico-chemical factors, but involves more complicated processes having an enzymic basis.

METHODS

The strain (6346) of *Bacillus subtilis* used, the techniques employed, the definitions adopted and the general approach to the problem have been described and discussed previously (Pollock 1961*b*). It should be particularly noted that the standard 'zero' reference time (0 hr.) from which all time periods are measured was taken as the point when the population density of growing cultures reached the equivalent of 0.1 mg. bacterial dry wt./ml. Unless stated otherwise this has also been the time when cells were induced to form penicillinase and α -glucosidase.

RESULTS

Effect of pH value

When the casein hydrolysate (CH) medium was supplemented with 2% (w/v) glucose, the organisms were found never to release more than a small fraction of their total penicillinase. Even after 6 hr. such glucose-grown organisms had liberated only 15% of their total penicillinase, in contrast to organisms grown in CH medium without glucose, where 40% or more of the enzyme was released into medium during the same period (see Pollock, 1961*b*). This effect was finally shown to be due mainly, if not entirely, to the lower pH value of the culture, which usually fell from 7.0 to 5.0-5.5 in glucose-containing cultures.

It was confirmed that the low amounts of extracellular penicillinase were not due to selective inactivation of exo-enzyme under the conditions associated with glucose-grown cultures. Concentrated dialysed supernatant fluid from a penicillin-induced culture at a final concentration of 13.5 units penicillinase/ml. was added to a 3 hr. uninduced culture of *Bacillus subtilis* growing in CH medium + 2% (w/v) glucose. Incubation continued for a further 2 hr. caused no significant loss of activity.

In the experiment summarized in Table 1 a culture grown in CH medium + 2% (w/v) glucose was split into two portions at 3 hr., and both portions incubated in large tubes at 35°, aerated by a flow of air through a sparger at 500 ml./min. In one portion the pH value was adjusted to pH 7.8 and kept constant thereafter for the 2 hr. of the experiment by an automatic titrator (Radiometer, Titrator TTTI, V. A. Howe and Co. Ltd., 46 Pembridge Road, W. 11) delivering minute quantities of N-NaOH into the culture continuously under the control of a pH meter. The total volume of NaOH thus added never increased the volume of the cultures by more than 3.5% and was ignored in comparing the results. A complementary experiment was also done without glucose in the medium, one of the two sister cultures being kept at pH 5.2 by automatic delivery of N-HCl while the control culture was left to drift towards alkalinity without adjustment. It can be seen that several times less enzyme was liberated in cultures at the lower pH values, regardless of whether or not glucose was present.

Effect of chloramphenicol

Addition of 40 μ g. chloramphenicol/ml. to a culture of *Bacillus subtilis* 6346 inhibited growth (opacity increase) and penicillinase production almost completely. The effect on enzyme liberation appeared at first to be variable. To obtain higher enzyme titres and more reliable results, some cultures were induced with cephalosporin C (1 μ g./ml.) and it was at once clear that significant enzyme release occurred

Table 1. *Effect of pH value on the liberation of penicillinase from penicillin-induced Bacillus subtilis 6346 growing in CH medium*

Conditions	Penicillinase activities (units/ml.)							
	pH value						Released during 3-5 hr. period	
	At 3 hr. before adjustment of (B)	At 5 hr.	At 3 hr.		At 5 hr.		Amount	% of total
			Total	Sn*	Total	Sn		
	Exp. 1.	In presence of 2% (w/v) glucose						
(A) No special control of pH value	5.2	5.4	30.96	2.92	31.2	3.8	0.88	2.8
(B) pH Adjusted to pH 7.8 at 3 hr. and thereafter kept constant	5.2	7.8	31.60	3.4	30.0	11.2	7.8	25.5
Exp. 2. Without added glucose								
(A) No special control of pH value	6.8	7.0	28.40	6.2	30.56	10.8	4.6	15.8
(B) Adjusted to pH 5.2 at 3 hr. and thereafter kept constant	6.8	5.2	28.76	6.0	27.76	7.6	1.6	5.6

* Sn = supernatant fluid.

in the presence of chloramphenicol; but no quantitative comparison with untreated cultures was possible because the total enzyme production was so much less than in the control. Repeated experiments with benzylpenicillin-induced cultures finally showed that chloramphenicol did in fact cause a significant and consistent inhibition of liberation of 30-50%; even in cultures where the slight difference in pH value from the control (due to growth inhibition and subsequently decreased metabolic drift towards alkalinity that is always shown by *B. subtilis* when growing in CH medium) was obliterated by the automatically titrating pH regulator, adjusted so that the pH value of the control culture was kept identical with that of the chloramphenicol-treated culture (measured independently with a second pH meter), as shown in Table 2. Neither earlier addition of chloramphenicol (namely at 2 hr.

Table 2. *Effect of chloramphenicol on the liberation of penicillinase from penicillin-induced Bacillus subtilis 6346 in CH medium*

Conditions	Penicillinase activities (units/ml.)						
	pH						Released into medium during 3-5 hr. period
	At 3 hr.	At 5 hr.	At 3 hr.		At 5 hr.		Amount
			Total	Sn*	Total	Sn	
Control (no addition)	7.0	7.65	17.6	3.6	21.2	6.8	3.2
Chloramphenicol 40 µg./ml. added at 3 hr.	7.0	7.65	—	—	20.4	5.2	1.6

* Sn = supernatant fluid.

instead of 3 hr.) nor raising its concentration to 100 $\mu\text{g./ml.}$ caused any significant change in the proportion of enzyme released.

Effect of temperature

Figure 1 shows the effect of temperature on penicillinase release from a culture previously induced with 0.25 mg. maltose/ml. and cephalosporin C (1 $\mu\text{g./ml.}$) treated with chloramphenicol (40 $\mu\text{g./ml.}$) at 3 hr. and immediately distributed in three lots in water baths maintained at 0°, 21° and 35°, respectively. The proportions of α -glucosidase released after 2½ hr. incubation at these three temperatures were found to be 36.3, 4.1 and 5.6 %, respectively. The cold treatment obviously

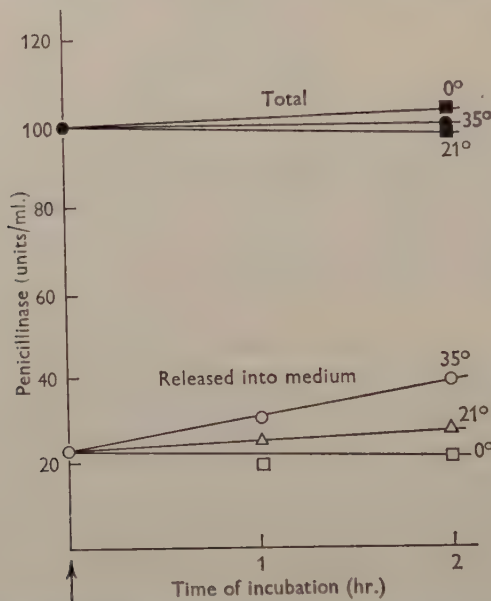


Fig. 1. The effect of temperature on the liberation in the presence of chloramphenicol of penicillinase from 3-hr. cells of *Bacillus subtilis* 6346, previously grown at 35° after induction with cephalosporin C (10 $\mu\text{g./ml.}$). 35° (circles: ●, ○); 21° (triangles: ▲, △); 0° (squares: ■, □); enzyme released into medium (open symbols: ○, △, □); total enzyme (filled-in symbols: ●, ▲, ■). Arrow indicates point of addition of chloramphenicol 40 $\mu\text{g./ml.}$

caused considerable cell damage, without, however, leading to penicillinase liberation, as long as the temperature was kept at 0°. When, however, the culture was returned to 35°, even brief cold treatment (e.g. 5 min. at 0°) resulted in substantial subsequent 'damage-release' of penicillinase, as shown in the first paper in this series (Pollock, 1961*b*; Fig. 5). In another experiment it was found that the penicillinase liberation increased further with temperatures above 35°; but at 45° this was always associated with considerable cell damage and could not therefore be regarded as physiological.

The rather striking dissociation between release of α -glucosidase and penicillinase at 0° in the experiment illustrated in Fig. 1 supports the original conclusion (Kushner & Pollock, 1961) that cell-bound penicillinase is normally fixed to particulate cell structure and does not exist 'free' in the cytoplasm (as appears to be the case with

α -glucosidase), and shows further that both the normal 'physiological' mechanism for the release of penicillinase and the secondary 'artefactual' release process, which under all other conditions so far investigated comes into effect following cell damage, are inoperative at 0° and therefore more likely to be dependent on some enzymic process in the cell rather than on diffusion or other purely physico-chemical factors.

Table 3. *Failure of high concentrations of salts to elute penicillinase from penicillin-induced Bacillus subtilis 6346 after 2 hr. treatment at 0°*

Treatment	α -Glucosidase (as μ mole <i>p</i> -nitrophenol/ml./hr.)			Penicillinase (units/ml.)		
	Total	In Sn	% in Sn*	Total	In Sn	% in Sn
Potassium phosphate (pH 7.0)						
10^{-3} M	3130	2030	65	17.0	0.48	2.8
5×10^{-2} M	3130	2500	80	17.8	0.68	3.8
2×10^{-1} M	3130	2500	80	16.1	0.76	4.7
Ammonium sulphate (pH 7.0)						
5×10^{-2} M	3130	2630	84	16.6	0.56	3.4
2×10^{-1} M	2970	2630	89	17.0	1.12	6.6

* Sn = supernatant fluid.

Effect of high salt concentrations

A 3 hr. culture of *Bacillus subtilis* 6346, previously induced with penicillin and maltose, was cooled to 0° and the organism resuspended in solutions of potassium phosphate and ammonium sulphate (pH 7.0) at concentrations of up to 0.2M for 2 hr. (at 0°, instead of 35°, to avoid 'damage-release'). Table 3 shows that negligible quantities of penicillinase ($>7\%$) were released by this treatment, which would be expected to elute a substantial fraction of enzyme, were the normal liberation process due to a desorption of protein bound to the cell by simple ionic linkages. This experiment shows the profound cell damage that occurred as a result of this treatment which led to the release of up to 90% of the α -glucosidase. It is also a further indication of the different manner in which the penicillinase is bound to the cells of *B. subtilis* as compared with *B. cereus*, where analogous salt treatment eluted nearly all the cell-bound β -penicillinase within a few minutes (Pollock & Kramer, 1958).

Possible role of enzymes

Effect of enzyme inhibitors. With 3 hr.-cultures of *Bacillus subtilis* 6346 growing in CH medium many of the substances and factors tested (1.6×10^{-3} M-*p*-chloromercuribenzoate; 0.05–1.0% (w/v) sodium deoxycholate; 8.3×10^{-4} M-8-hydroxyquinoline) increased penicillinase liberation but rapidly led to cell damage (α -glucosidase leak) so that their action on physiological release could not be assessed. Other substances added (5×10^{-3} M-Na₃N₃; 10^{-3} M-2:4-dinitrophenol; CuSO₄, 100 μ g./ml.; 0.02M sodium succinate; mercaptoethanol, 0.5 mg./ml.; carbamylcholine, 0.1 mg./ml.; histamine phosphate, 0.1 mg./ml.) had no detectable effect. Ethylenediamine tetra-acetic acid (10^{-3} M), in the presence of chloramphenicol (40 μ g/ml.), had a slight inhibitory effect on penicillinase liberation (up to 40% from cells of a cephalosporin C-induced culture in some experiments), but this was not consistently repeatable. A similar

variable but even slighter inhibitory effect was shown by low doses of ultraviolet radiation in the absence of chloramphenicol. High concentrations of Mg^{++} (0.05M- $MgSO_4$) which had been shown to have a very striking inhibitory effect on the release of the enzyme from membrane preparations or damaged cell suspensions (Kushner & Pollock, 1961) had no action whatever in cultures growing normally in CH medium.

Proteinases. The specific penicillinase-liberating action of proteinases on sucrose-treated suspensions of *Bacillus subtilis* 6346 (Kushner & Pollock, 1961) and the possibly enzymic nature of the liberation process suggested that this might normally involve the action of some proteolytic enzyme or esterase, either by direct detachment of the enzyme by hydrolysis of a peptide bond which linked it to some surface structure, or by destruction (solubilization) of a protein responsible for its fixation. Attempts to inhibit the hypothetical esterase involved, by addition of diisopropyl-fluorophosphate (DFP) and heparin (0.1%) were unsuccessful. Concentrations of DFP up to a nearly saturated solution (addition of 1 drop of pure DFP in 10 ml. of culture, repeated twice during the 2 hr. experiment) had no significant effect on liberation of penicillinase from cephalosporin C-induced cells of a culture previously treated with chloramphenicol (40 $\mu g./ml.$).

Table 4. *The effect of crude dialysed extracts of organisms from old Bacillus subtilis 6346 cultures on release of α -glucosidase and penicillinase from cells of a maltose- and penicillin-induced homologous B. subtilis culture in casein hydrolysate (CH) medium*

Time	Volume (ml.) of extract* added (at 3 hr.) to 10 ml. culture	Bacterial suspension concentration (equiv. mg. dry wt./ml.)	α -Glucosidase (μ mole <i>p</i> -nitrophenol/ ml./hr.)			Penicillinase units/ml.)		
			Total	In Sn†	% in Sn	Total	In Sn	% in Sn
3 hr. (before addition of extract)	—	0.85	4310	47	1.1	21.6	4.8	22.2
5½ hr.	0	2.10	4130	65	1.6	20.8	7.6	36.5
	0.023	2.25	4000	108	2.7	21.6	8.3	38.5
	0.067	2.44	4500	338	7.5	21.2	9.5	44.8
	0.2	2.10	4810	1275	26.5	22.0	12.2	55.3
	0.5	1.04	4900	3090	63	20.4	15.6	76.5
	1.8	0.37	5100	4560	89.5	21.6	13.3	61.7

* The extract was diluted so that 1.0 ml. contained material originally derived from the organisms of 10 ml. of a 24 hr. culture. Further details, see text.

† Sn = supernatant fluid.

Crude cell extracts. Extracts were prepared from organisms of an uninduced culture of *Bacillus subtilis* 6346, by resuspending the organisms at ten times their original concentration in 10^{-2} M-potassium phosphate buffer (pH 7.0) and crushing them in a Hughes press at -30° . The supernatant fluid after centrifugation of the disrupted suspension at 20,000 *g* for 20 min. was dialysed against 10^{-3} M-potassium phosphate (pH 7.0) and concentrated by freeze-drying. These extracts had enzyme-like properties which appeared to be similar to those of the 'autolysin' of *B. subtilis* strain H described by Nomura & Hosoda (1956). When added to cultures of *Bacillus subtilis* 6346 in CH medium the extracts consistently caused cell damage, the extent of

which depended upon the quantity of extract and the age of the culture from which it was prepared. Severe damage resulted in cell lysis. Less severe damage was shown by leakage of α -glucosidase and increase in penicillinase liberation; but it was not possible to dissociate these two effects by diluting the extract. A typical experiment is illustrated in Table 4 where increasing quantities of an extract prepared from a 24 hr. culture were added to 10 ml. samples of a 3 hr.-culture previously induced with maltose and penicillin. The effects on opacity increase and release of the two induced enzymes into the medium were followed during incubation for a further $2\frac{1}{2}$ hr. It can be seen: (a) only the highest concentration of extract caused obvious cell lysis (decrease in suspension opacity) although $1/27$ of this concentration caused a significant α -glucosidase leak of 7.5%; (b) liberation of penicillinase was increased by addition of extract very roughly in proportion to the extent of α -glucosidase release; there was no concentration of extract which stimulated penicillinase release without significantly increasing the release of α -glucosidase above the basal value of 1.6% shown by the control culture. Extracts from the organisms of young cultures were very much less active in causing cell damage than extracts from organisms of older cultures. For instance, the degree of cell damage, as measured by % α -glucosidase leakage, produced by extract of a given dry weight from organisms of 3 hr.- or 6 hr.-cultures, could be evoked by approximately one-fiftieth of that quantity of extract prepared from 20 hr.-cultures. The autolytic factor or factors concerned were completely inactivated by boiling for 30 min. It is clear that the factor(s) was being produced in quantity only in old cultures, where some slight activity was also demonstrable in concentrated dialysed supernatant fluid as well as in the organisms themselves.

In addition, extracts of organisms from 20 to 24 hr. cultures were found to contain proteinase activity, whose action in liquefying gelatin and destroying α -glucosidase was almost completely inhibited by 5×10^{-3} M-DFP. For this reason this proteinase activity can be almost certainly rejected as participating in normal penicillinase liberation or in the 'damage-release' effect of the cell extracts described above, because these processes are not affected by DFP. It is possible that some sort of lysozyme-like enzyme, known to be formed by certain strains of *Bacillus subtilis* (Richmond, 1959*a*, *b*) might be partly responsible for the lytic action of these extracts; but it is rather unlikely on *a priori* grounds that such an enzyme plays a part in normal penicillinase liberation. In any case, egg-white lysozyme by itself had a completely different action: at concentrations of 1 μ g./ml. and above, it caused rapid lysis of 3-hr. cultures, whereas at 0.4 μ g./ml. and below there was no detectable effect whatever (no growth inhibition, no leak of α -glucosidase, no stimulation of penicillinase liberation). All further attempts, by graded ammonium sulphate precipitation, zone electrophoresis and column fractionation, to isolate a specific penicillinase-liberating factor from these extracts, by separation from other material which might be interfering with its action or causing cell damage, have so far failed.

DISCUSSION

The problem of exo-enzyme liberation from bacteria has been approached in the absence of any real knowledge about the nature of the structural barriers or physical or chemical entities which may obstruct free diffusion of penicillinase from a cell after the enzyme is formed. It may well be that *Bacillus subtilis* 6346 penicillinase

is a special case and that the mechanism of its liberation is unrelated to the mechanism acting on other exo-enzymes (e.g. α -amylase from the same strain) where little or no enzymic activity is found to be associated with the cell under any conditions (Dr R. A. Darrow; personal communication) and where, therefore, the liberation process may be linked with the formation of the enzymes (either *de novo* or from an enzymically inactive high molecular weight precursor).

The classical concept of the bacterial cell as consisting of cytoplasm surrounded by a selectively impermeable membrane enveloped by a relatively permeable cell wall (see Salton, 1960; McQuillen, 1960) rather implied that the essential process involved in enzyme release must be its passage through the cytoplasmic membrane. Such a picture may be mistaken. The cytoplasmic membrane has chemical and anatomical reality and there is good reason for believing it to be mainly responsible for the selective permeability of the bacterial cell. But little is known about how exo-enzymes are formed and nothing about the site of their synthesis. It is possible that the process of specific polypeptide chain production which leads to their formation occurs outside the main permeability barrier of the cell. In any case, the classical picture of a continuous and undifferentiated cytoplasmic membrane may be a gross oversimplification. In *Bacillus subtilis* itself there are now indications of a sort of rudimentary endoplasmic reticulum (Glauert, Brieger & Allen, 1961) which might play a part in enzyme liberation. Finally, the barrier to large molecules offered by the cell wall is quite unknown, though Mitchell & Moyle (1959) and Mitchell (1961) have produced evidence which suggests that in *Staphylococcus aureus* and *Escherichia coli* it may not be negligible.

The present work permits only tentative and limited conclusions; but they may be useful in forming a basis for further studies. Assuming that the population of organisms in the culture is homogeneous with respect to enzyme release it can be accepted that at least a fair proportion of the penicillinase originally bound to cells of *Bacillus subtilis* is released into the medium by a process not involving marked cell damage. The complete inhibition of liberation at 0°, the zero order kinetics, the partial inhibition by chloramphenicol and the failure to elute enzyme from the cells by high concentrations of salts, all suggest that the process may be enzymic rather than physico-chemical. Chloramphenicol would be expected to inhibit the formation but not the functioning of such an enzyme system and would therefore cause only partial inhibition of the processes governed by the enzyme; this was what was observed. But it must be admitted that the failure to increase the inhibition of enzyme release by adding chloramphenicol earlier on during cultivation, when the cell content of the hypothetical enzymes responsible for liberation might be expected to be lower, does not support this interpretation. Conclusive evidence about the nature of the enzyme or enzymes involved has not been obtained.

Cell extracts of organisms from old cultures of the homologous strain of *Bacillus subtilis* 6346 increased the rate of penicillinase release from organisms from 3-hr. cultures. The main question is, what part, if any, substances present in these extracts may play in normal physiological liberation of the enzyme. But this stimulatory effect on penicillinase release was always associated, *pari passu*, with cell damage, as indicated by a leakage of α -glucosidase which did not occur in cultures which were spontaneously liberating penicillinase in the normal way. It did not prove possible, by a variety of techniques, to dissociate the two effects. Such

'autolytic' extracts might be expected to contain many different enzymes; but the only activity present which was specifically identified was that of a DFP-sensitive proteinase. This could be rejected as a possible agent for normal penicillinase liberation because of the latter's insusceptibility to inhibition by DFP. This negative finding does not completely exclude the possibility that a proteinase of some sort plays a part in penicillinase liberation, since DFP-insensitive examples of this kind of enzyme (e.g. cathepsin B) have been reported (see Fruton, 1960). In any case, the possibility of some specific penicillinase-liberating factor being present in these extracts cannot be excluded simply by negative results which, it might still be argued, may only reflect the difficulty of reproducing an enzymic effect which is suspected of occurring somewhere within the cell envelope, by adding the enzyme artificially from the outside. The great decrease in liberation of enzyme at a pH value (5.5) at which most cell enzymes might be expected to function poorly, is also consistent with the hypothesis that the phenomenon has an enzymic basis.

Although gross cell damage appears to be excluded, it can always be argued that minor degrees of damage (e.g. the initial steps of a chain of metabolic events finally leading to observable damage and lysis) are, nevertheless, an essential preliminary to exo-enzyme release. This possibility, which is really the essence of the view put forward by Nomura, Hosoda & Yoshikawa (1958) is made less likely by the insusceptibility to the inhibitory effect of high Mg^{++} concentration, and the absence of concomitant α -glucosidase release which characterize 'physiological' liberation of penicillinase and thus distinguish it qualitatively from 'damage-release'.

It might, perhaps, be preferable to consider the possibility that liberation of penicillinase is a reflexion of some relatively non-specific process of change in the biochemical, chemical and physical properties of the cell envelope, affecting the extents to which several other substances, as well as penicillinase, were bound to it. Many bacterial exo-enzymes appear during the later stages of batch growth of *Bacillus* sp. (see Pollock, 1961*a*). The reason for this is not yet understood. It is striking, for instance, to note that the period in a batch culture when penicillinase is being liberated corresponds closely to that during which α -amylase is appearing in the medium. It would be useful, therefore, to know whether generalized changes do occur in the cell wall and other superficial cell structures over this period.

It seems clear that in *Bacillus subtilis* 6346 cell-bound penicillinase is normally fixed to solid cell structure, possibly in part to the cytoplasmic membrane itself. Although only a proportion ($> 50\%$) of this cell-bound penicillinase was conclusively shown by antiserum neutralization experiments (Kushner & Pollock, 1961) to be on the outside of the membrane, the results as a whole are consistent with it all being there. It is possible that the membrane itself may be the site of its formation. If this be so, the mechanism of exo-enzyme release may involve detachment of enzyme from some superficial structure (which the penicillinase-releasing effect of trypsin suggests may be protein in nature, Kushner & Pollock, 1961), followed by its passage through the cell wall. This appears, at least for the moment, to be a useful working hypothesis to guide further studies.

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The Genetic Relationship and Phenotypic Expression of Mutations Endowing *Pneumococcus* with Resistance to Erythromycin

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SUMMARY

Five spontaneous erythromycin resistance mutations arising independently in a pneumococcal strain have been studied. Three distinct levels of resistance are represented by these mutants (0.1, 1.0, and 10.0 $\mu\text{g.}$ erythromycin per ml.). The mutations conferring resistance can be transferred to the sensitive parental strain through transforming DNA preparations. The transfer is discrete, in that the full level of resistance of the donor strain is always conferred upon the recipient. The length of time required for phenotypic expression of a mutation acquired by transformation depends on the particular marker.

A mutation in a given strain may either be replaced by or combine with a different mutation transferred from a donor DNA preparation. In the case of combination, the DNA of the recombinant is capable of transferring each of the mutations as well as the entire complex of mutations possessed by the recombinant. The frequency of transfer of the complex demonstrates the degree of linkage of the separable mutations. A group of mutations in a given recombinant strain may either display antagonistic, synergistic or non-synergistic effects on the phenotype.

Reverse mutations towards erythromycin-sensitivity generally involve alteration at the originally mutated sites, or at very closely linked sites.

INTRODUCTION

In recent years the fine structure of genetic material has been investigated in great detail in a number of micro-organisms (Pontecorvo, 1952; Pritchard, 1955; Benzer, 1955; Demerec, 1956). These investigations have shown that the particular segment of genetic material concerned with a specific function of the organism is capable of undergoing numerous mutations situated at different sites separable by genetic recombination. The order of these mutations in a linear array can generally be determined by the additivity (or near-additivity) of the frequencies of recombination observed to occur in crosses between the various pairs.

The analysis of genetic fine structure has been extended to deoxyribonucleic acid (DNA) transforming factors (Hotchkiss & Evans, 1958; Lacks & Hotchkiss, 1960; Ravin, 1960; Ephrati-Elizur, Srinivasan & Zamenhof, 1961; Rotheim & Ravin, to be published). The molecule of DNA has been found to be large and complex enough not only to bear regions concerned with different functions of the organism (Hotchkiss & Marmur, 1954; Goodgal & Herriott, 1957), but also numerous mutable sites

separable by recombination within each region (Ephrussi-Taylor, 1951; Ravin, 1960; Lacks & Hotchkiss, 1960; Ephrati-Elizur *et al.* 1961; Rotheim & Ravin, to be published). Recombinable mutations borne by the same molecule of DNA can often be arranged in a linear order (Hotchkiss & Evans, 1958; Ravin, 1960; Lacks & Hotchkiss, 1960; Ephrati-Elizur *et al.* 1961).

This report concerns the genotypic and phenotypic analysis of five independent mutations conferring resistance to erythromycin on pneumococci. It has been known (Haight & Finland, 1952*b*) that mutants arising spontaneously generally resist only a relatively low concentration of erythromycin; strains enhanced in their level of resistance can be obtained by repeated selections at gradually increasing concentrations of the antibiotic. Such step-by-step accretion of resistance is similar to that observed with penicillin (Demerec, 1945; Hotchkiss, 1951). The mutations described in this paper were found either to replace or combine with each other in transformation reactions. Some combinations led to enhanced levels of resistance, although non-synergistic and antagonistic interactions were also observed with certain groups of combined mutations. All of the mutations, however, like those affecting other functions previously studied, behaved as linked factors, that is, as factors occupying different sites of the same molecule of DNA.

Most effective against Gram-positive cocci and diphtheria bacilli (Haight & Finland, 1952*a*), erythromycin may be either bacteriostatic or bactericidal, depending upon the concentration. At the concentrations used in the present experiments, its action was principally killing. Like penicillin, its bactericidal action requires active multiplication of the treated strain (Haight & Finland, 1952*c*).

METHODS

Organisms and genetic markers. The erythromycin sensitive strain of *Pneumococcus* used in these experiments is Rx, a capsule-deficient mutant derived from a type III encapsulated strain (SIII-N) that had been previously produced by transformation (Ravin, 1959). The Rx strain does not form visible or microcolonies when plated on blood agar containing more than 0.01 μ g. erythromycin per ml. (Table 1). Green (1957) obtained a number of spontaneous erythromycin resistant mutants by plating 10^9 Rx bacteria on a blood agar medium containing 1 μ g. erythromycin per ml. Twenty large colonies appeared after 72 hr. incubation at 37°. Strains derived from three of the twenty mutant colonies isolated by Green were examined in the studies reported here; they contain, respectively, the mutations *ery-r2*, *ery-r3* and *ery-r5*. The strains derived from two of the other colonies isolated by Green, originally resistant to at least 1 μ g. erythromycin per ml., subsequently reverted to the wild-type erythromycin sensitive condition during the transfer of stocks. They reverted before it was possible to determine their genotypic relationship to the three mutants just mentioned. These reverted strains are referred to as E21-rev. and E24-rev.

Subsequently, two additional spontaneous mutants of independent origin were obtained. One of these was a mutant colony obtained by plating a culture of strain SIII-1b (Ephrussi-Taylor, 1951) on blood agar containing 0.1 μ g. erythromycin per ml. Upon isolation, this strain was found, by the method of direct plating described below, to be incapable of growing in the presence of erythromycin at concentrations greater than 0.1 μ g. per ml. The genetic factor responsible for the

erythromycin resistance of this strain was transferred to the Rx strain by exposing the latter to the DNA extracted from a culture of mutant cocci. This transformed strain of Rx proved to have the same level of resistance as that of the original SIII-1b mutant. The mutation contained in this strain is referred to as *ery-r6*. The other spontaneous mutation was obtained by plating a culture of R6R, a derivative of the capsule-deficient strain R36A (Ravin, 1960), on a blood agar medium containing 0.1 μ g. erythromycin per ml. One of the colonies that developed upon incubation was found to consist of cocci capable of resisting a much higher concentration of erythromycin than that used for selecting the mutant. The mutation responsible for this high level of resistance, referred to as *ery-r7*, was subsequently transferred by means of transformation to the Rx strain. The Rx transformants thus produced possessed the same level of resistance as that of the original R6R mutant (Table 1).

Table 1. *Maximum level of resistance of mutants bearing different ery-r mutations, including several recombinant types, and the time required for phenotypic expression of resistance newly acquired by transformation*

Category	Marker (s)	Max. level of resistance (μ g. erythromycin./ml.)	Concentration of erythromycin (μ g./ml.) used to challenge trans-formants	Time (hr.) at which ability to resist challenging concentration is expressed in all trans-formants
1. Mutations of strain Rx	<i>ery-r6</i>	0.1	0.075	1
	<i>ery-r2</i>	1.0 \pm 0.2	0.25	1
	<i>ery-r3</i>	1.0 \pm 0.2	0.25	1
	<i>ery-r5</i>	1.0 \pm 0.2	0.25	1
	<i>ery-r7</i>	10.0 \pm 5.0	0.1	3
			5.0	> 5
2. Recombinations in strain Rx	<i>ery-r2-r6</i>	5.0	—	—
	<i>ery-r3-r6</i>	15.0	—	—
	<i>ery-r2-r3</i>	40.0 \pm 10.0	—	—
	<i>ery-r6-r7</i>	10.0 \pm 5.0	—	—
	<i>ery-r5-r7</i>	10.0 \pm 5.0	—	—
	<i>ery-r2-r3-r5</i>	15.0 \pm 5.0	—	—
3. Mutations in other strains	<i>ery-r6</i> in strain SIII-1b	0.1	—	—
	<i>ery-r7</i> in strain R6R	10.0 \pm 5.0	—	—
4. Wild-type (Rx)	<i>ery-s2-s3-s5-s6-s7</i>	0.01	—	—

The variability indicated for certain mutations and recombinations expresses approximately the highest and lowest maxima observed in a series of similar tests.

At various times the *ery-r* markers have been transferred into closely related strains (e.g. SIII-1, SIII-2, Rz, etc.; see Ravin, 1959) which differ from Rx only in the possession of a different mutation within the type III capsule locus (Ravin 1960). The DNAs obtained from such transformed strains have been used in some of the transformation experiments described below.

An additional genetic factor was occasionally also present in the particular donor strain from which the transforming DNA was prepared. This additional factor was

str-r1 (Ravin, 1956; Bryan, 1961), which confers the ability to resist up to 6000 μ g. streptomycin per ml. Thus, DNA preparations generally contained, in addition to the *ery-r* factor being investigated, either *str-r1* or *caps*⁺, the wild-type allele of the mutated factor (*caps*⁻) responsible for the capsule deficiency in the Rx strain (Ravin, 1960), or sometimes both factors. Neither of these factors is linked to any of the *ery-r* factors described in these experiments (Ravin, 1960). The additional genetic factor served as a control in certain experiments; the successful transfer of this factor showed that the failure to obtain alterations in the level of erythromycin resistance of the treated recipient strain could not be ascribed either to incompetence of the recipient culture or inactivation of the transforming preparation.

The genetic factors in the sensitive Rx strain which are homologous to those in the respective erythromycin-resistant and streptomycin-resistant mutant strains are symbolized as follows: *ery-s*, *str-s*.

It should be pointed out that a factor (*dep*) linked to the streptomycin-resistance marker *str-r1*, which lowers the frequency of genetic integration of the latter into the genomes of certain recipient strains, is of no significance in the experiments to be reported here since the *dep* factor has no effect when transferred into Rx bacteria (Green, 1959; M. B. Rotheim, to be published).

Transforming preparations. The method of preparing the DNAs used in these experiments is essentially identical to that described by Ephrussi-Taylor (1951). The DNAs were preserved by solution (at concentrations from 0.01 to 0.1 %, w/v) in a physiological saline solution (0.15 M-NaCl) buffered with phosphate (pH 7) and storage at 4°. Saturating concentrations of DNA were used in all experiments.

Media. Three media, originally described by Ephrussi-Taylor (1951), were used in these experiments. Medium 1, which consists of 1 % (w/v) Neopeptone (Difco), 0.4 % (w/v) yeast extract (Difco) and 0.86 % (w/v) NaCl in demineralized water, was used for growing cultures to be used as sources of transforming DNA. High yields of bacteria were obtained by the addition of an excess of glucose (0.63 %, w/v, in final concentration) and intermittent additions of N-NaOH to neutralize the acid produced in the course of growth.

Medium 2 was the standard plating medium. In addition to the ingredients present in medium 1, it contained 1.5 % (w/v) agar (Difco or BBL), 0.033 % (w/v) glucose and 2 % (w/v) sterile defibrinated sheep blood (Cappel Labs). Glucose (sterilized separately by filtration) and sterile blood were added to the rest of the autoclaved and cooled medium just before the pouring of plates. Plates were poured and, before use, they were dried overnight by incubation in a forced air (CENCO) incubator at 37°. To media to be used for the assay of antibiotic-resistant mutants or transformants were added, at the time of addition of blood and glucose, an appropriate amount of the stock solution of the antibiotic in question. The usual concentrations of antibiotic used in the screening of resistant bacteria produced by transformation were 0.1, 0.25 and 5.0 μ g. erythromycin per ml. and 100 μ g. streptomycin per ml. In certain transformation experiments, other concentrations were used, and these will be specified below. The antibiotic products used were obtained as erythrocin lactobionate (Abbott) and streptomycin sulfate (Lilly).

Medium 3 consisted of inorganic salts, Neopeptone (Difco), an extract of bovine serum, charcoal-adsorbed yeast extract, and a growth-limiting concentration of glucose dissolved in glass-distilled demineralized water. Transformations and stock

transfers were carried out in this medium. For the transfer of stocks 0.1 ml. of sterile defibrinated rabbit blood (Cappel Labs.) was added per 5 ml. of medium. Cultures in any of these media were incubated at 37°.

Assay of antibiotic resistance. The maximum level of resistance of an isolated strain was determined in one of two ways: (A) Direct plating—A sample is taken from a medium 3 culture and appropriately diluted in 1% (w/v) Neopeptone (Difco); similar samples from the dilution are spread by means of a bent glass rod over the surface of plates of previously dried medium 2, some of which are devoid of antibiotic ('plain agar') while others constitute a series of increasing concentration of antibiotic. That concentration of antibiotic above which the final number of colonies appearing is significantly less than that observed in plain agar is arbitrarily chosen as the maximum level of resistance of the strain being tested. (B) Streak testing—in the cases where one must test a large number of colonies arising from a culture treated with transforming DNA, the colonies are first transferred by isolating them individually with a needle and spreading over the surface of plain agar. Some of the growth arising on these plates is then transferred, either by streaking with a needle or by replicating a velvet impression (Lederberg & Lederberg, 1952), on to a series of plates, each containing a different concentration of antibiotic. Similar streaks are made at the same time to plain agar as a control for the viability of the growth being tested. While streak testing has certain advantages for the examination of a large number of strains, it has the disadvantage that the application of dense populations of bacteria on to a given region of an antibiotic-containing medium occasionally gives spurious results. Some physiological mechanism allows the crowded population, or part of it, to resist higher concentrations of antibiotic than the individual cocci are capable of resisting in isolation. Streak tests thus often need to be rechecked and verified by the use of the method of direct plating.

Cultures that have been exposed to transforming DNA were analysed in the following way. Appropriately diluted samples of such cultures were plated on plain agar, on agar containing various concentrations of erythromycin, and, where necessary, on agar containing 100 µg. streptomycin per ml. The titres of total viable bacteria (obtained from the plain agar plates) and of bacteria resistant to the given concentrations of antibiotic were determined from the counts of colonies formed after 48 or 72 hr. of incubation (in any case, at a time beyond which continued incubation brings forth no more colonies). The frequency of resistant transformants, expressed as the fraction of the total number of viable cocci capable of resisting a given concentration of antibiotic, can then be calculated. The maximum level of resistance of colonies growing in agar containing a certain concentration of antibiotic was determined by isolating such colonies and testing the isolates by either direct plating or streak testing.

Transformation. In a transformation experiment a recipient strain of a given genetic constitution was exposed to the DNA extracted from a donor strain of different genetic constitution. Recombinant bacteria containing some of the genetic factors of the donor and some of the recipient were sought among the progeny of the treated recipient bacteria. In a real sense, therefore, a transformation experiment is equivalent to a 'cross' between two individuals of different genotype.

The transformation was carried out in one of the following ways. (A) Long-term experiment: in this type of experiment, the recipient cells are exposed to DNA for

a relatively long period of time lasting many generations. The transforming DNA remains in contact with the cells during the time in which they are becoming competent to react with DNA, and for several generations after the effective contacts have occurred. It is a simple procedure to use when certain qualitative results are desired (such as the production of any recombinants at all from a given 'cross'). The procedure consists in transferring into medium 3 containing the appropriate DNA plus 0.2 % (w/v) bovine serum albumin (Armour Fraction V), a small number of cocci of the desired recipient strain from a medium 3 culture which has just stopped growing. The initial bacterial density is arranged to be about 500 per ml.; there is no appreciable lag following such a transfer. After 7 to 11 generations of growth ($3\frac{1}{2}$ to $5\frac{1}{2}$ hr. after inoculation), the cocci become competent and react with the DNA present in the medium (Ravin, 1956). The culture is then plated after growth is terminated by the exhaustion of glucose (10 hr. after inoculation). The time interval between effective contact of competent cocci with transforming DNA and the plating of the culture is, in every case, sufficient to permit full phenotypic expression of the newly acquired genetic determinants (Table 1). However, the time interval is large enough to permit some change in the proportion of transformant types due to differential selective action against them by the medium. This possibility has been checked by reconstruction experiments using mixtures of transformant types, and, where necessary, as will be discussed below, short-term experiments are conducted to determine the proportions of transformant types found immediately after phenotypic expression. (B) Short-term experiment: in this type of experiment, DNA is allowed to act upon competent recipient bacteria for only a brief interval of time (10–20 min. = $\frac{1}{3}$ – $\frac{2}{3}$ generation time). Competent bacteria are usually obtained by transfer of an inoculum, similar to that described above, into medium 3 containing 0.2 % (w/v) bovine serum albumin. A volume of 0.1 ml. of the appropriate DNA is added at $4\frac{1}{2}$ to 5 hr. after inoculation, and the action of the DNA is terminated by the addition 10–20 min. later of 0.1 ml. of a Mg^{++} -activated DNase solution (Ravin, 1956). The treated culture is plated either at various hourly intervals after exposure to DNA or at a time (generally 2 hr. after exposure) when it is known that phenotypic expression is completed. In a few cases, it was necessary to perform a series of experiments over a period of time with a recipient strain using cultures of reproducible levels of competence. For this purpose, a modification of the 'freezing' technique of Fox & Hotchkiss (1957) was successfully employed. About 10^7 pneumococci previously grown in medium 3 were inoculated into a flask containing 45 ml. of medium 3 plus serum albumin (0.2 %, w/v) and incubated in a water bath at 37° . At 15 min. intervals during growth, 0.5 ml. amounts of the culture are withdrawn and added to a series of tubes containing 1.5 ml. of preserving medium, previously chilled in an ice-bath. The contents of each tube is mixed by shaking and immediately frozen in a deep-freeze at -80° . Preserving medium was prepared by mixing one part of sterile 2 % (w/v) Neopeptone (Difco) containing 10^{-3} M-CaCl₂ with one part of sterile 20 % (w/v) glycerol (sterilized by filtration). One tube from each series prepared in this manner was tested with an active transforming DNA preparation, to determine the level of competence of cells in each series. Those series not possessing sufficiently high levels of competence were discarded; those series containing competent cells retained their high level of competence in the frozen state for at least a few weeks. Frozen recipient cocci to be transformed are thawed in the cold (4 – 5°),

and then to 0.2 ml. of cell suspension are added 1.8 ml. of medium 3 containing 0.2 % (w/v) serum albumin and 0.1 ml. of the desired DNA. The mixture is incubated at 30–32° (optimal temperature for adsorption of DNA) for 20 min. The DNA is then inactivated by addition of 0.1 ml. of Mg^{++} -activated DNase. The treated suspension is then incubated at 37° (optimal temperature for growth) for a period of time sufficient to allow phenotypic expression to occur before assay of the culture.

RESULTS

Levels of resistance of various mutants and of several recombinant types. The maximum level of erythromycin resistance of each of the five spontaneous mutants was determined by the method of direct plating. The results are recorded in Table 1. It will be noted that the mutation *ery-r6* confers the lowest level of resistance, that the mutations *ery-r2*, *ery-r3* and *ery-r5* confer a similar intermediate level of resistance, and that the mutation *ery-r7* confers the highest level of resistance. The difference between the *ery-r6* level and that of *ery-r2* is about tenfold; the difference between the *ery-r2* level and that of *ery-r7* is also roughly tenfold.

Some physiological variability was observed in the maximum level of resistance obtainable by a given strain. This was found in slight differences in the resistance of a given strain using cultures grown at different times. An indication of the extent of the variability is given in Table 1. The resistances characteristic of the three levels have remained distinct, however, such that, for example, the resistance conferred by a mutation of the intermediate level has never descended as low as the level characteristic of the low level nor risen as high as the level characteristic of the high level.

Table 1 also records the maximum levels of erythromycin resistance possessed by recombinant strains obtained by transformation experiments to be described below. It will suffice to point out here certain antagonistic and synergistic actions between mutations. For example, when the *ery-r2* and *ery-r3* mutations are present in the same strain, they confer together about twenty times more resistance than the sum of their individual resistances. When the *ery-r5* mutation is combined with the *ery-r2* and *ery-r3* mutations, the level of resistance of the resulting triply marked bacterium drops by slightly more than half of the level possessed by a strain bearing only the *ery-r2* and *ery-r3* mutations. Thus, the mutations *ery-r2* and *ery-r3* are synergistic in combination, whereas the mutation *ery-r5* is antagonistic to the action of the *ery-r2-r3* combination. The *ery-r7* mutation, on the other hand, behaves neither synergistically nor antagonistically when recombined with either *ery-r5* or *ery-r6*. The recombinant (possessing both *ery-r7* and either *ery-r5* or *ery-r6*) exhibits a level of resistance which is indistinguishable from that of the strain bearing only the *ery-r7* factor.

Transfer of mutations by DNA-transforming preparations and their behaviour following transfer. The antibiotic-sensitive Rx strain was transformed in short- and long-term experiments with the DNAs obtained from strains bearing the *str-r1* mutation and one of the *ery-r* mutations. The concentration of antibiotic in the plates used to determine the presence of antibiotic-resistant transformants was always considerably less than the maximum level characteristic of the mutation in question. The reason for this procedure was twofold. Some transformants may be

lost if challenged with the maximum concentration of antibiotic they are genetically capable of withstanding due either to: (1) physiological variability, as a result of which some bacteria in a given culture always possess a lower level of resistance than the average level inherited by the strain, or (2) insufficient time allowed for the phenotypic expression of newly transformed bacteria. The amount of time required for phenotypic expression is measured by the interval between the time of exposure of the recipients to transforming DNA and the time when the fraction of viable bacteria capable of forming colonies in a given concentration of antibiotic reaches a maximum. In this respect, it was found, for example, that the time at which all of the transformants requiring *ery-r7* can be recovered depends on the concentration of antibiotic with which the bacteria are challenged. When the challenging concentration of erythromycin was 0.1 $\mu\text{g. per ml.}$, 3 hr. were required to obtain all of the *ery-r7* transformants. On the other hand, when the challenging concentration was 5 $\mu\text{g. per ml.}$, over 5 hr. of growth were required for all of the *ery-r7* transformants to be detected. It is entirely possible that the expression of the *ery-r7* mutation is all-or-none as Fox (1959) found for a streptomycin resistance mutation, but that the probability that an unexpressed transformant will express the phenotype corresponding to its newly acquired genotype is inversely related to the rate of killing, which is greater for higher challenging concentrations of antibiotic. In any event it is clear that, while 3 hr. were required for phenotypic expression of the resistance conferred by mutation *ery-r7* to 0.1 $\mu\text{g. erythromycin per ml.}$, only 1 hr.

Table 2. *Discreteness of transfer of erythromycin resistance mutations in short-term transformation experiments*

1. *ery-r6 str-s1* \times DNA *ery-r6 str-r1*; plated 1 hr. after exposure to DNA

Frequency of transformants producing colonies in presence of		Of 118 colonies tested from 0.075 $\mu\text{g. ery./ml.}$		
100 $\mu\text{g. strep./ml.}$	0.075 $\mu\text{g. ery./ml.}$	No. able to resist	$\mu\text{g. ery./ml.}$	
			0.1	0.25
1.3 $\times 10^{-3}$	1.2 $\times 10^{-3}$	118	+	—
		0	+	+

2. *ery-s7 str-s1* \times DNA *ery-r7 str-r1*; plated 3 hr. after exposure to DNA

Frequency of transformants producing colonies in presence of		Of 45 colonies tested from 0.05 $\mu\text{g. ery./ml.}$			
100 $\mu\text{g. strep./ml.}$	0.05 $\mu\text{g. ery./ml.}$	No. able to resist	$\mu\text{g. ery./ml.}$		
			1	15	30
5.9 $\times 10^{-4}$	4.5 $\times 10^{-4}$	45	+	+	—
		0	+	—	—

3. *ery-s3 str-s1* \times DNA *ery-r3 str-s1*; plated 2 hr. after exposure to DNA

Frequency of transformants producing colonies in presence of		Of 120 colonies tested from 5.0 $\mu\text{g. ery./ml.}$			
0.1 $\mu\text{g. ery./ml.}$	5.0 $\mu\text{g. ery./ml.}$	No. able to resist	$\mu\text{g. ery./ml.}$		
			0.1	1	5
4.5 $\times 10^{-3}$	< 10^{-5}	120	+	+	—
		0	+	+	+
		0	+	—	—

was needed for the completion of phenotypic expression of the resistance conferred by mutations *ery-r6* and *ery-r3* to 0.075 and 0.25 μ g. erythromycin per ml. respectively. The time required for expression of the maximal level of resistance that a transformant is genetically capable of withstanding depends, therefore, on the particular *ery-r* marker being considered (Table 1).

Consequently, when the sensitive Rx strain is treated with the DNA containing the *ery-r2* marker, for example, the treated population is challenged with only 0.25 μ g. erythromycin per ml., although the maximum level of resistance conferred by this marker is four times as great. Furthermore, this challenge is carried out at a time when it is known that all transformants have expressed their ability to resist the challenging concentration of erythromycin (Table 1). The same challenging concentration was found useful for the *ery-r3* and *ery-r5* markers. However, 0.075 μ g. erythromycin per ml. was the challenging concentration used for detecting transformants acquiring the *ery-r6* marker, while 0.1 μ g. erythromycin per ml. was the concentration used for detecting transformants acquiring the *ery-r7* marker.

In either long- or short-term experiments, it was found that each *ery-r* marker was transferred as a discrete unit. The only class of antibiotic-resistant transformants detectable was that having the same level of resistance as that of the donor strain. This conclusion is based on the fact that, when Rx cells treated with DNA bearing a given *ery-r* marker were challenged by plating in media containing a concentration of erythromycin far below that which the donor strain was capable of resisting, all of the colonies subsequently arising were successfully replicated or streaked on to agar containing the maximum concentration of erythromycin which the donor was capable of resisting, but not on to agar containing a higher concentration. Thus, transformants were not found to possess intermediate or higher levels of resistance than the donor strain. Typical results for the markers *ery-r3*, *ery-r6* and *ery-r7* are shown in Table 2.

Table 3. *Recombinations between various ery-r mutations*

Mutant recipient strain	Mutant donor strain				
	-r2	-r3	-r5	-r6	-r7
-r2	—	-r2-r3 (40)	—	-r2-r6 (5)	s2-r7 (10)
-r3	-r2-r3 (40)	—	—	—	s3-r7 (10)
-r5	—	—	—	—	r5-r7 (10)
-r6	-r2-s6 (1) -r2-r6 (5)	-r3-s6 (1) -r3-r6 (15)	-r5-s6 (1)	—	r6-r7 (10)

Transformants with increased level of resistance are sought by challenging on agar containing a concentration of erythromycin sufficient to prevent growth of dense populations of recipient strain (0.25 μ g./ml. -r6; 5 μ g./ml. -r2, r3 and r5). Value indicated in parentheses is maximum level of resistance of recombinant. Negative result (—) means that number of colonies observed on agar containing the challenging concentration of erythromycin is no greater than the number arising spontaneously (i.e. in absence of DNA). Indicated genotypes of recombinants are verified in subsequent experiments.

Recombination between mutations. A given mutant may be treated with the DNA extracted from another mutant, and one can determine if transformants are produced having a higher level of resistance than that of the recipient strain. Following long-term exposure of the recipient strain to the particular DNA, one challenges by plating on agar containing a concentration of erythromycin somewhat greater than

the maximum concentration tolerated by the recipient strain. The actual challenging concentration employed is one just sufficiently high to prevent even slight or feeble growth by dense populations of untreated recipient bacteria spread over the surface of agar. Table 3 records the results of all possible 'crosses' involving the various mutants as donors and the *ery-r2*, *ery-r3*, *ery-r5* and *ery-r6* strains as recipients.

It will be recalled that the *ery-r2*, *-r3*, and *-r5* mutations confer indistinguishable levels of resistance upon a sensitive strain. Although phenotypically similar, however, the three mutations are genotypically distinct. For example, while no mutant strain can be raised in its level of resistance by autologous DNA, recombinants having increased resistance are produced in the 'cross' *ery-r2* × *ery-r3*. The outcome is identical regardless of how the 'cross' is carried out: mutant *ery-r2* + DNA *ery-r3*, or (the reciprocal 'cross') mutant *ery-r3* + DNA *ery-r2*. Thus, *ery-r2* is different from *ery-r3*. It will be noted that, while the challenging concentration for selecting recombinants in these crosses is 5 µg. erythromycin per ml., all of the selected transformants are capable of resisting as much as 40 µg. erythromycin per ml. The mutant *ery-r5*, on the other hand, when treated with either DNA *ery-r2* or DNA *ery-r3* did not give rise to cocci with an increased level of resistance to erythromycin. Similarly, neither *ery-r2* nor mutant *ery-r3* produced cocci with increased resistance when treated with DNA *ery-r5*. This is presumptive evidence that *ery-r5* is distinct from both *ery-r2* and *ery-r3*; confirmatory evidence is obtained from experiments to be described below in which *ery-r5* is raised in its level of resistance by treatment with DNA containing both the *ery-r2* and *ery-r3* markers.

When the mutant *ery-r6*, which has a lower level of resistance than any of the other strains, serves as recipient, one may select for transformants capable of resisting at least the challenging concentration normally employed for detecting transformants acquiring the donor marker, and then one may determine, by subsequent replication or streak-testing, whether any of the colonies growing in the presence of the challenging concentration of erythromycin can grow in the presence of even higher concentrations. Thus, it is found that when, for example, mutant *ery-r6* is treated with DNA *ery-r2*, two classes of transformed pneumococci are obtained: those resisting up to 1 µg. erythromycin per ml., and, hence, identical to organisms containing *ery-r2*; and those resisting up to 5 µg. erythromycin per ml. The latter class is presumed to be the recombinant type *ery-r2-r6*. The presence of both markers in such bacteria can be proved by experiments described in the next section. It will also be noted that the recombinant type *ery-r2-r6* is obtained when mutant *ery-r2* is treated with DNA *ery-r6* and then challenged with 5 µg. erythromycin per ml. (Table 3). It is significant, furthermore, that when either mutant *ery-r3* or mutant *ery-r5* is treated with DNA *ery-r6*, no transformant class can be detected that is capable of resisting a higher concentration of erythromycin than the recipient strain (Table 3).

The DNA of mutant *ery-r7* has been tested on each of the other mutant strains, and in every case, regardless of the *ery-r* mutation possessed by the recipient strain, transformants enhanced in their level of resistance were detected by plating in agar containing a concentration of erythromycin just sufficiently high to block the growth of untreated recipient bacteria. By streak-testing, it was found that the transformants thus produced exhibited the same phenotype as that of the *ery-r7* strain (Table 3).

Linkage of erythromycin resistance mutations. Having obtained enhancement of resistance by treating one mutant strain with the DNA of another, it was of interest to determine the genetic relationship of the mutations involved in such a cross. When, as in the case of crosses involving *ery-r7* mutant as donor, only one transformant class was obtained, corresponding in phenotype to that of the donor, it could be supposed that *ery-r7* replaces every one of the other mutations in transformation reactions. Further investigation proved, however, that this was not the case. A DNA preparation was made from a single transformant selected in each one of the following crosses: *ery-r2* \times *ery-r7*; *ery-r3* \times *ery-r7*; *ery-r5* \times *ery-r7*; *ery-r6* \times *ery-r7*. Two of the DNAs, those prepared from transformants issuing from the former two crosses, behaved as though only the *ery-r7* mutation was contained in the transformed strain. This was determined by exposing the sensitive Rx strain to the DNA in a short-term experiment and then selecting for bacteria on agar containing 0.1 μ g. erythromycin per ml. All of the selected bacteria proved to possess the *ery-r7* phenotype. However, the DNAs produced from transformants obtained in the latter two crosses produced two classes of resistant bacteria when used to treat the sensitive Rx strain. The phenotype of one of the classes corresponded to that of the *ery-r7* strain, but the phenotype of the other class corresponded to that of the other mutant involved (*ery-r5* or *ery-r6*). For example, in one experiment a transformed strain possessing the phenotype of the *ery-r7* mutant was selected following a cross between recipient *ery-r5* and donor *ery-r7*. The DNA of this selected transformant was prepared and tested in a short-term exposure of the sensitive Rx strain. Cocci capable of producing colonies in the presence of 0.1 μ g. erythromycin per ml. were selected. Of 106 such colonies tested, 83 were able to resist a maximum erythromycin concentration of 1 μ g./ml. while 23 were able to resist no more than 10 μ g./ml. Thus two phenotypic classes were obtained; one corresponding to that of *ery-r5-s7*, the other corresponding to that of either *ery-s5-r7* or *ery-r5-r7*. The former class is by far the more abundant. The conclusion to be drawn from the ensemble of these results is that the *ery-r7* mutation is capable of replacing either the *ery-r2* or *ery-r3* mutation, but it can combine with either the *ery-r6* or *ery-r5* mutation. Whether *ery-r7* replaces or combines with another mutation, however, the recombinant possesses the *ery-r7* phenotype. It is altogether possible that the *ery-r7* mutation can combine with the *ery-r2* and *ery-r3* mutations, but the lack of synergism produced with the *ery-r7* mutation makes it difficult to determine. If, for example, *ery-r7* can replace as often as it can combine with one of these mutations, a randomly isolated transformant (obtained in the cross *ery-r2*, or *ery-r3*, \times *ery-r7*) is just as likely to possess the *ery-s2-r7* genotype as the *ery-r2-r7* genotype. Since further experiments established the linkage of the *ery-r2*, *ery-r3*, *ery-r5* and *ery-r6* mutations, it is clear that the *ery-r7* mutation is linked to all of them.

It will be recalled that enhancement of resistance was obtained in a cross involving the *ery-r2* and *ery-r3* mutations, and in this case the transformed class possessed a maximum level of resistance forty times greater than that of the untreated recipient. In this case, it may be supposed that the transformed strain with the enhanced resistance possesses two mutations, the original mutation of the recipient plus that transferred from the donor via the transforming DNA preparation. Proof that this view is in fact correct may be obtained by extracting the DNA from the trans-

formant supposedly bearing both mutations (*ery-r2* and *ery-r3*) and then briefly exposing a sensitive strain to this DNA. Three genotypic classes are expected, corresponding to bacteria containing *ery-r2* alone, bacteria containing *ery-r3* alone, and bacteria containing both *ery-r2* and *ery-r3*. Furthermore, the frequency of transformed bacteria acquiring both mutations relative to the frequency of bacteria acquiring only a single mutant marker provides a means of determining how closely the two mutations are linked.

By this procedure, it has been ascertained that the two mutations are indeed linked, i.e. behave as if borne by the same molecule of transforming DNA. In Table 4 are recorded the results of a typical experiment in which the sensitive Rx strain is treated with DNA from the transformed strain *ery-r3-r2 str-r1*. It will be noted that the frequency of transformants capable of resisting 0.25 µg. erythromycin per ml. is similar to the frequency of transformants capable of resisting 100 µg.

Table 4. *Classes of transformants produced following short-term exposure of sensitive Rx strain to DNA from the presumptive Rx ery-r2-r3 str-r1*

Period of exposure of competent Rx cells to DNA: 20 min.
Period of growth following exposure to DNA: 8 hr.

1. Frequency of transformants producing colonies in presence of:

Erythromycin		Streptomycin
0.25 µg./ml.	5 µg./ml.	(100 µg./ml.)
3.9×10^{-4}	5.1×10^{-5}	3.3×10^{-4}

2. Tests of isolated colonies

Isolated from agar containing	Capable of growing on transfer to agar containing	Proportion
0.25 µg. ery./ml.	1.0 µg. ery./ml.	1.0
	5.0 µg. ery./ml.	1.5×10^{-1}
	100 µg. strep./ml.	6.9×10^{-3}
100 µg. strep./ml.	0.25 µg. ery./ml.	5.8×10^{-3}

3. Further test of 10 colonies

Isolated from agar containing 0.25 µg. ery./ml. but incapable of growing at concentrations greater than 1.0 µg. ery./ml.

Number producing transformants capable of resisting 5 µg. ery./ml. when treated with:

DNA <i>ery-r2</i>	DNA <i>ery-r3</i>	Both DNAs
4	6	0

streptomycin per ml. Of the colonies that develop in agar containing 0.25 µg. erythromycin per ml., only about one in 150 is found to be capable of resisting 100 µg. streptomycin per ml. A similar proportion of colonies that develop in agar containing 100 µg. streptomycin per ml. is found to be capable of resisting 0.25 µg. erythromycin per ml. This proportion is taken as an index of the frequency of random penetration of competent recipient cocci by two independent ('non-linked' transforming molecules. It will be noted that the frequency of transformants that develop in agar containing 5 µg. erythromycin per ml. is greater than one-tenth the frequency of transformants that develop in agar containing 0.25 µg. erythromycin per ml. (precisely, the ratio of these two frequencies is 1.3×10^{-1}). Similarly,

if one tests a random sample of colonies that develop in agar containing 0.25 μ g. erythromycin per ml., roughly one in six (precisely, 1.5×10^{-1}) are found to be capable of resisting 5 μ g. erythromycin per ml. The class of transformants capable of resisting 5 μ g. erythromycin per ml., presumably possessing both the *ery-r2* and *ery-r3* factors, is obviously occurring more frequently than would be expected on the basis of the random penetration of two independent transforming factors. Furthermore, the maximum level of resistance of this class of transformants, as subsequently determined, was found to be 40 μ g. erythromycin per ml., which is the phenotype of the donor strain. Similarly, it was found that the transformants that cannot resist this high concentration of erythromycin can resist at most 1 μ g. erythromycin per ml., which is the phenotype of either the *ery-r2* or *ery-r3* strains. It can be shown, moreover, that these transformants are of two genetic types, one corresponding to *ery-r2*, the other to *ery-r3*. For example, in the experiment recorded in Table 4, 10 of the transformants appearing in agar containing 0.25 μ g. erythromycin per ml. that subsequently were found to be unable to resist 5 μ g. erythromycin per ml., were subjected to the following test. One culture of each transformant was treated with DNA *ery-r2 str-r1*, and another culture rendered competent under similar conditions was treated with DNA *ery-r3 str-r1*. Streptomycin resistance transformations were induced in all of the cultures. However, six of the transformants were enhanced in their resistance to erythromycin only when exposed to DNA containing the *ery-r3* factor, while four were enhanced only when exposed to DNA containing the *ery-r2* factor. The former corresponded, therefore, to cocci containing the *ery-r2* factor, while the latter corresponded to cocci containing the *ery-r3* factor. Thus, transformants having the *ery-r2-s3* and *ery-s2-r3* genotypes were recovered in this experiment. In summary, the *ery-r2* and *ery-r3* factors can be brought together and can be separated by genetic recombination; when together, they behave as though borne by the same molecule of transforming DNA.

Experiments of similar design were carried out to determine the linkage of other pairs of erythromycin resistance markers. For example, linkage of *ery-r2* to *ery-r6* was demonstrated in the following ways. First of all, long-term exposure of mutant *ery-r6* to DNA containing the factors *ery-r2* and *str-r1* reveals that *ery-r6* is apparently replaced by *ery-r2* most of the time (Table 5A). That the *ery-r2-r6* recombinant occurs is indicated by the existence of a class of transformant that can resist a higher concentration of erythromycin than can *ery-r2*; this class can resist 5 μ g-erythromycin per ml. The existence of the recombinant is also readily demonstrable by treating mutant *ery-r2* with DNA containing the *ery-r6* and *str-r1* markers (Table 5B). For about 20 transformants acquiring the *str-r1* factor, one transformant appears that is capable of resisting as much as 5 μ g. erythromycin per ml. The DNA of one such transformant was prepared and used to treat the sensitive Rx (*ery-s2-s6*) strain in a short-term experiment. The results (Table 5C) reveal that three phenotypic classes of transformants are obtainable: one corresponding to the *ery-r6* phenotype, one corresponding to the *ery-r2* phenotype, and one corresponding to the *ery-r2-r6* phenotype. The proportions of these classes were found to be approximately 0.6:3:1. Repeat experiments have confirmed the interesting finding that the *ery-r6-r2* transformant occurs more frequently than the *ery-r6-s2* transformant, which is corroborative evidence of the strong linkage of *ery-r6* to *ery-r2*.

Furthermore, *ery-r6* appears to be even more tightly linked to *ery-r3*. This is revealed by the fact that in five independent long-term experiments in which strain *ery-r3* was exposed to DNA containing the factors *ery-r6* and *str-r1*, no transformants were found capable of resisting 5 μ g. erythromycin per ml. although transformants acquiring the *str-r1* factor were abundant (Table 6B). Moreover, in the reciprocal cross (strain *ery-r6* treated with DNA *ery-r3 str-r1*) the recombinant *ery-r3-r6* also occurred rarely although the replacement of *ery-r6* by *ery-r3* occurred

Table 5. *Recombination between the mutations ery-r6 and ery-r2*

A. Long-term exposure of mutant *ery-r6* to DNA *ery-r2 str-r1*

	Frequency of transformants producing colonies in presence of		
	0.25 μ g. ery./ml.	5 μ g. ery./ml.	100 μ g. strep./ml.
<i>ery-r6</i> + no DNA	$< 10^{-7}$	$< 10^{-7}$	$< 10^{-7}$
<i>ery-r6</i> + DNA	$1.0 \times 10^{-4}\dagger$	3.1×10^{-5}	1.2×10^{-3}

B. Long-term exposure of mutant *ery-r2* to DNA *ery-r6 str-r1*

	Frequency of transformants producing colonies in presence of	
	5 μ g. ery./ml.	100 μ g. strep./ml.
<i>ery-r2</i> + no DNA	$< 10^{-7}$	$< 10^{-7}$
<i>ery-r2</i> + DNA	$1.0 \times 10^{-6}\dagger$	2.0×10^{-5}

C. Short-term exposure of sensitive Rx strain to DNA *ery-r2-r6*§

	Frequency of transformants producing colonies in presence of				
	0.05 $\mu\text{g. ery./ml.}$	0.25 $\mu\text{g. ery./ml.}$	5.0 $\mu\text{g. ery./ml.}$		
Rx + no DNA	1.0×10^{-5}	$< 10^{-5}$	$< 10^{-5}$		
Rx + DNA	3.5×10^{-4}	3.5×10^{-4}	2.4×10^{-5}		
	Maximum level of resistance ($\mu\text{g. ery./ml.}$) of isolated colonies				
		No. resisting			
Source of colonies: growing in presence of	Total no. tested	0.1	0.25	5	15
0.05 $\mu\text{g./ml.}$	64	9	41	14	0
0.25 $\mu\text{g./ml.}$	188	—	145	43	0
5.0 $\mu\text{g./ml.}$	15	—	—	15	0

† Of 35 colonies isolated all could grow at an erythromycin concentration of 0.25 μ g./ml. while 8 could grow at a maximum erythromycin concentration of 5 μ g./ml.

‡ Of 8 colonies isolated, all could grow at a maximum concentration of 5 μ g. ery./ml.

§ Source of DNA being strain capable of resisting 5 μ g. erythromycin per ml. obtained in experiment B.

almost as frequently as does the acquisition of the *str-r1* marker (Table 6 A,C). The rare *ery-r3-r6* recombinant obtained in the reciprocal cross was found to resist as much as 15 μ g. erythromycin per ml. In accord with its presumed genotype, the DNA of the *ery-r3-r6* recombinant produces three phenotypic classes in a sensitive recipient strain: one corresponding to the *ery-r6* mutant, one corresponding to the *ery-r3* mutant, and one corresponding to the *ery-r3-r6* recombinant (Table 6C). It is interesting to note, however, that these three classes were produced, respectively, in the proportion 18:1:6. Again, as in the case of the DNA of the *ery-r2-r6* recombi-

nant, the transfer of both mutated sites into the recipient sensitive strain occurred more frequently than did the single transfer of one of the mutated sites. In the present case, however, it was the *ery-r3* mutation that was transferred least frequently, whereas in the previous case it was the *ery-r6* mutation that was transferred least frequently.

Table 6. *Recombination between the mutations ery-r6 and ery-r3*

A. Long-term exposure of mutant *ery-r6* to DNA *ery-r3 str-r1*

	Frequency of transformants producing colonies in presence of	
	0.25 μ g. ery./ml.	100 μ g. strep./ml.
<i>ery-r6</i> + no DNA	$< 10^{-6}$	$< 10^{-6}$
<i>ery-r6</i> + DNA	$1.1 \times 10^{-5}\dagger$	1.9×10^{-5}

B. Long-term exposure of mutant *ery-r3* to DNA *ery-r6 str-r1*

	Frequency of transformants producing colonies in presence of	
	5 μ g. ery./ml.	100 μ g. strep./ml.
<i>ery-r3</i> + no DNA \ddagger	$< 10^{-7}$	$< 10^{-7}$
<i>ery-r3</i> + DNA \ddagger	$< 10^{-7}$	3.9×10^{-5}

C. Short-term exposure of sensitive Rx strain to DNA *ery-r3-r6§*

	Frequency of transformants producing colonies in presence of		
	0.1 μ g. ery./ml.	0.25 μ g. ery./ml.	5.0 μ g. ery./ml.
Rx + no DNA	$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$
Rx + DNA	1.7×10^{-4}	2.9×10^{-5}	$< 10^{-5}$

Maximum level of resistance (μ g. ery./ml.) of isolated colonies

Source of colonies: growing in presence of 0.1 μ g./ml.	Total no. tested	No. resisting				
		0.1	0.25	5	15	30
	76	55	3	0	18	0

\dagger Of 52 colonies isolated, 51 could grow at a maximum erythromycin concentration of 1 μ g./ml., while 1 could grow at a maximum concentration of 15 μ g./ml.

\ddagger Typical result of 5 independent experiments.

\S Source of DNA being strain capable of resisting 15 μ g. erythromycin per ml. obtained in experiment A.

In a similar fashion, it has been determined that the site of the *ery-r5* mutation either overlaps that of *ery-r6*, or is very closely linked to it. When strain *ery-r5* was treated with DNA *ery-r6 str-r1*, no transformants were found capable of resisting 5 μ g. erythromycin per ml. (Table 7B). In the reciprocal cross (strain *ery-r6* treated with DNA *ery-r5 str-r1*), transformants capable of resisting 1 μ g. erythromycin per ml. occurred almost as frequently as transformants acquiring the *str-r1* factor (Table 7A). Furthermore, DNA extracted from one of the erythromycin-resistant transformants, which are identical in phenotype to *ery-r5* cocci, showed in this strain, at least, no evidence of bearing the *ery-r6* marker. On the sensitive Rx strain, only one class of transformants was produced, namely, those capable of resisting at most 1 μ g. erythromycin per ml. (Table 7C). Thus, the *ery-r5* mutation was shown to be capable of replacing the *ery-r6* mutation.

The relation of ery-r5 to ery-r2 and ery-r3. On the basis of the results just discussed, it is possible to conclude:

- (1) that the erythromycin resistance mutations *ery-r2*, *-r3*, *-r5*, *-r6* and *-r7* are borne by the same molecule of transforming DNA;
- (2) that the sites of the following mutations are separable by genetic recombination: *ery-r2* and *ery-r3*; *ery-r2* and *ery-r6*; *ery-r3* and *ery-r6*; *ery-r6* and *ery-r7*.
- (3) that the site of the *ery-r6* mutation is very closely linked to the site of the *ery-r3* mutation, more so than to the site of the *ery-r2* mutation, and it either overlaps or is closely linked to the site of the *ery-r5* mutation.

Table 7. *Recombination between mutations ery-r6 and ery-r5*

A. Long-term exposure of mutant *ery-r6* to DNA *ery-r5 str-r1*

	Frequency of transformants producing colonies in presence of	
	0.25 μ g. ery./ml.	100 μ g. strep./ml.
<i>ery-r6</i> + no DNA	$< 10^{-6}$	$< 10^{-6}$
<i>ery-r6</i> + DNA	$1.4 \times 10^{-5}\dagger$	2.0×10^{-5}

B. Long-term exposure of mutant *ery-r5* to DNA *ery-r6 str-r1*

	Frequency of transformants producing colonies in presence of	
	5 μ g. ery./ml.	100 μ g. strep./ml.
<i>ery-r5</i> + no DNA \dagger	$< 10^{-7}$	$< 10^{-7}$
<i>ery-r5</i> + DNA \dagger	$< 10^{-7}$	4.4×10^{-5}

C. Short-term exposure of sensitive Rx strain to DNA *ery-(r6)r5§*

	Frequency of transformants producing colonies in presence of	
	0.1 μ g. ery./ml.	
Rx + no DNA	$< 10^{-5}$	
Rx + DNA	$5.6 \times 10^{-3}\parallel$	

\dagger Of 60 colonies isolated, all were capable of growing at a maximum erythromycin concentration of 1 μ g./ml.

\ddagger Typical result of 5 independent experiments.

\S Source of DNA is a strain capable of resisting 1 μ g. ery./ml. obtained in experiment A.

\parallel Of 205 colonies isolated, all could grow at a maximum erythromycin concentration of 1 μ g./ml.

The relation of the *ery-r5* mutation to the *ery-r2* and *ery-r3* mutations is problematic. It was noted that the mutation *ery-r5* did not recombine with either *ery-r2* or *ery-r3* to produce a recombinant possessing a significantly greater level of resistance. This fact suggests that either the *ery-r5* mutation recombined with *ery-r2* (or *ery-r3*) to yield an *ery-r2-r5* (or *ery-r3-r5*) recombinant having no enhanced power of resistance (or else a lower level of resistance), or that the *ery-r5* mutation is allelic to the *ery-r2* and *ery-r3* mutations in overlapping both of them. The first evidence bearing on this question suggested that the former explanation is correct. When the mutant bearing the *ery-r5* mutation was treated with DNA from a strain containing the *ery-r2-r3* and *str-r1* markers, two classes of erythromycin-resistant transformants were recovered in agar containing 5 μ g. erythromycin per ml.: a class the maximum level of resistance of which was 15 μ g. erythromycin per ml., and a

class the maximum level of resistance of which was about 40 μ g. erythromycin per ml. The former was considerably more frequent, since nine of eleven independent transformants belonged to this class. On the contrary, when the *ery-r2* or *ery-r3* mutant was treated with this DNA, the only class of transformants recovered was one capable of resisting a maximum of 40 μ g. erythromycin per ml. This class corresponds to the expected *ery-r2-r3* recombinants. These findings demonstrated that the mutation *ery-r5* was indeed distinct from both *ery-r2* and *ery-r3*. They indicate, moreover, that the replacement of *ery-r5* by *ery-r2-r3* occurred less frequently than recombination between *ery-r5* and *ery-r2-r3*. The phenotype of the presumed *ery-r2-r3-r5* recombinant has a lower level of resistance than that of the *ery-r2-r3* donor, and it is therefore suggestive that the mutation *ery-r5* in conjunction with either *ery-r2* or *ery-r3* had a lower level of resistance than a mutant bearing only one of the muta-

Table 8. *Short-term exposure of Rx ery-s2-s3-s5 str-s1 to DNA ery-r2-r3-r5 str-r1*

A. Frequency of transformant classes as a function of time following exposure to DNA

Incubated cells in presence of albumin for 4 hr. 10 min.; added DNA for 20 min.; added DNase; then plated at hourly intervals.

Time after addition of DNase ...	1 hr.	2 hr.	3 hr.	4 hr.
Total no. cocci per ml.	1.5×10^6	5.1×10^6	2.3×10^7	8.9×10^7
Freq. of transformants producing colonies in 100 μ g. strep./ml.	5.2×10^{-6}	6.3×10^{-6}	4.1×10^{-6}	4.7×10^{-6}
Freq. of transformants producing colonies in 0.25 μ g. ery./ml.	7.1×10^{-6}	1.2×10^{-5}	5.7×10^{-6}	1.0×10^{-5}
Freq. of transformants producing colonies in 5 μ g. ery./ml.	<u>6.6×10^{-7}</u>	1.2×10^{-6}	<u>1.0×10^{-7}</u>	7.6×10^{-7}

(Underlining means frequency determination is based on colony count of < 10.)

B. Test of *str-r* transformants selected on 100 μ g. strep./ml.

Total no. colonies tested:	621
Total no. colonies that can resist at least 0.25 μ g. ery./ml.	3
Proportion of <i>str-r</i> transformants that can resist ery.	4.8×10^{-3}

C. Test of *ery-r* transformants selected on 0.25 μ g. ery./ml.

Total no. colonies tested:	714
Total no. colonies that can resist at least 5 μ g. ery./ml.	94
Proportion of <i>ery-r</i> transformants that can resist 5 μ g. ery./ml.	1.3×10^{-1}
Total no. of <i>ery-r</i> transformants found to resist at least 5 μ g. ery./ml.	94
No. of these transformants found to resist at most 15 μ g. ery./ml.	94
No. of these transformants found to resist at most 40 μ g. ery./ml.	0

D. Test of *ery-r* transformants selected on 5 μ g. ery./ml.

Total no. colonies tested:	15
Total no. colonies resistant to at most 15 μ g. ery./ml.	14
Total no. colonies resistant to at most 40 μ g. ery./ml.	1

E. Analysis of 24 *ery-r* transformants incapable of resisting 5 μ g. ery./ml.

No. of transformants enhanced in erythromycin resistance:	
by exposure to DNA <i>ery-r2 str-r1</i> , but not to DNA <i>ery-r3 str-r1</i>	6*
by exposure to DNA <i>ery-r3 str-r1</i> , but not to DNA <i>ery-r2 str-r1</i>	18**
by exposure to neither DNA	0

* 2; transformants further tested; genotypes of both: *ery-r3-r5*.

** 7; transformants further tested; genotypes of all: *ery-r2-r5*.

tions. This hypothesis would account for the absence of transformants enhanced in their level of resistance when the mutant bearing the *ery-r5* marker is crossed with a mutant bearing either the *ery-r2* or *ery-r3* marker.

An investigation was undertaken of the DNA extracted from one of the presumed *ery-r2-r3-r5* recombinants (i.e. a transformant resisting at most 15 μ g. erythromycin per ml.) into which the *str-r1* marker was subsequently transferred. The sensitive Rx strain was exposed to this DNA in a short-term experiment. If *ery-r5* is separable by recombination from *ery-r2* and *ery-r3*, then one should expect to recover the following genotypic products: *ery-s2-s3-r5*, *ery-r2-s3-s5*, *ery-s2-r3-s5*. In addition of course, genotypes containing two, and even three, mutant markers should be produced. The actual results obtained reveal a more complex situation (Table 8). Transformants capable of resisting at most 1 μ g. erythromycin per ml. occur about six times more frequently than transformants capable of resisting a higher concentration of erythromycin (Table 8A). The vast majority of the latter are phenotypically identical to the donor strain (i.e. *ery-r2-r3-r5*), which resists at most 15 μ g. erythromycin per ml. (Table 8C). Transformants capable of resisting 40 μ g. erythromycin per ml. (i.e. corresponding to the genotype *ery-r2-r3-s5*) are rare, but have occasionally been detected (Table 8D). Incidentally, the relatively high frequency of joint transfer of the *ery-r2*, *-r3*, and *-r5* factors is corroborative evidence of the linkage of these three factors (Table 8 B, C). As for the transformants capable of resisting only a low concentration of erythromycin, they were subjected to further analysis (Table 8E). They were first isolated and grown in medium 3. Samples from the same competent culture of a given isolated transformant were then exposed separately to DNA *ery-r2 str-r1* and to DNA *ery-r3 str-r1*. These treated cultures were then challenged on agar containing 5 μ g. erythromycin per ml. and on agar containing 100 μ g. streptomycin per ml. The appearance of a significant number of colonies in streptomycin-containing agar reveals that the DNAs are active and that the cultures were competent when exposed to the DNAs. In every case where these conditions were fulfilled, a significant number of colonies appeared on agar containing 5 μ g. erythromycin per ml. as a result of exposure to either the DNA bearing the *ery-r2* marker or to the DNA bearing the *ery-r3*, but not both. Thus, among the transformants resisting a low concentration of erythromycin obtained in the original treatment of the sensitive Rx strain with DNA *ery-r2-r3-r5 str-r1*, the genotype *ery-s2-s3-r5* is rare, if it occurs at all. These transformants could have, however, one of the following genotypes: *ery-r2-s3-s5*, *ery-s2-r3-s5*, *ery-s2-r3-r5*, *ery-r2-s3-r5*. Bacteria having one of the latter two genotypes could possibly have the same level of resistance as bacteria having one of the former two genotypes, as mentioned above. However, bacteria having the genotype *ery-r2-s3-r5* could be distinguished from those having the genotype *ery-r2-s3-s5* by the fact that the latter would produce recombinants capable of resisting 40 μ g. erythromycin per ml. following exposure to DNA *ery-r3*, whereas the former would produce recombinants capable of resisting at most 15 μ g. erythromycin per ml. In a similar fashion, bacteria possessing the genotype *ery-s2-r3-s5* could be distinguished from those possessing the genotype *ery-s2-r3-r5* by determining the level of resistance of recombinants produced following exposure to DNA *ery-r2*. Therefore, following the exposure to DNA *ery-r2* and to DNA *ery-r3* the colonies appearing in agar containing 5 μ g. erythromycin per ml. were isolated and tested for their maximum level of resistance. The outcome of such an analysis

(Table 8E) reveals that the transformants capable of resisting only a low concentration of erythromycin are of the following genotypes, in the proportions indicated: 3 *ery-r2-s3-r5*, 1 *ery-s2-r3-r5*. The *ery-r2-s3-s5* and *ery-s2-r3-s5* genotypes apparently occur infrequently.

The principal conclusion to be drawn from this study, therefore, is that the *ery-r5* mutation once linked to the *ery-r2* and *ery-r3* mutations by genetic recombination can be separated from those mutations only with great difficulty. The surprising finding is that the association of *ery-r5* with *ery-r2-r3* occurs considerably more frequently than would be expected from the rarity of dissociation of *ery-r5* from *ery-r2-r3*. A number of attempts have been made to eliminate the *ery-r5* mutation from the *ery-r2-r3-r5* strain by replacing it with its *ery-s5* allele. This was done by long-term exposure of the strain to each of the following DNAs: *ery-s2-s3-s5 str-r1*, *ery-r2-s3-s5 str-r1*, *ery-s2-r3-s5 str-r1*, *ery-r2-r3-s5 str-r1*, and by challenging samples of the treated strain with 40 µg. erythromycin per ml. or 100 µg. streptomycin per ml. Although acquisition of the *str-r1* marker could be demonstrated, no recombinants having the *ery-r2-r3-s5* genotype could be detected by this method. One may conclude either that a challenge of 40 µg. erythromycin per ml. is too great for *ery-r2-r3-s5* transformants to withstand only a few generations after transformation, or that replacement, within the *ery-r2-r3-r5* complex, of the *ery-r5* mutated site by the corresponding unmutated allele is extremely rare. The latter explanation seems more likely, since some *ery-r2-r3-s5* transformants can be detected in long-term experiments in which sensitive Rx cocci (*ery-s2-s3-s5*) are treated with DNA *ery-r2-r3-s5* and challenged with 40 µg. erythromycin per ml. It is true, however, that the recovery of such transformants is more efficient when a lower challenging concentration of erythromycin is used.

Reversions of the ery-r3 and ery-r5 mutations. In the periodic transfer of stock mutant cultures, it was noted that certain mutants often reverted to the wild-type (erythromycin-sensitive) condition. This was noted when samples of the cultures were plated on plain agar and on agar containing a concentration of erythromycin which the originally isolated mutants were able to resist. A far greater number of colonies appearing on plain agar than on erythromycin agar suggested that reversions had occurred and had been selected for. That this was indeed the case was shown by the fact that a large proportion of the colonies appearing on plain agar were identical to wild-type in their level of resistance to erythromycin, and that the cocci in such colonies (or in wild-type cultures) had a slight but definite selective advantage when mixed with the original mutant type and grown in media in the absence of erythromycin. The most commonly reverting mutant cultures were those of *ery-r3* and *ery-r5*. The mutation *ery-r2* was very stable, and indeed, no case of a reversion has been noted despite numerous transfers of several different clones bearing this marker during the more than 2 years in which this marker has been studied. Furthermore, the mutant culture *ery-r2-r3* often drops in its level of resistance from 40 to 1 µg. erythromycin per ml.; but when it does so, it is found that the mutation at the *ery-r3* site has reverted, while the mutation at the *ery-r2* site has persisted. The method of determining the site of reversion will be described below. It should be stated at this point, however, that despite the known revertibility of the *ery-r3* and *ery-r5* mutations, the recombinant *ery-r2-r3-r5* is quite stable. A search for a reverted type having the genotype *ery-r2-r3-s5*, having a higher level of resistance

than the triply mutant type, has been fruitless. Finally, it should be stated that at least in the routine inspection of transferred stocks, the *ery-r6* and *ery-r7* mutations have not been found to undergo reversions to the wild-type condition.

There are essentially four possibilities of accounting for a reversion in one of the erythromycin-resistant strains. The first possibility is that a true back-mutation occurs, in the sense that the mutated site of the DNA molecule reverts to the original physico-chemical configuration that existed in the parental wild-type erythromycin-sensitive strain. The second possibility is that, in a reversion, the mutated site undergoes a change in physico-chemical configuration, which is not to that of the parental wild-type molecule but which, nevertheless, confers the same sensitivity to erythromycin as does the wild-type configuration. For reasons to be discussed below, it is not easy to distinguish experimentally between these first two possibilities, and therefore the term 'back-mutation' will be applied to both in the sense of referring to involvement of the mutated site in reversion. The third possibility is that, in a reversion, a suppressor mutation arises linked to the site of the erythromycin resistance mutation, and as a consequence, the resistance normally conferred by the latter is suppressed. The fourth possibility is similar to the third, except that the suppressor is unlinked to the erythromycin resistance mutation. For singly marked *ery-r3* and *ery-r5* mutants, the third and fourth possibilities may be tested: (a) by treating one of the reverted strains with the DNA of the wild-type sensitive Rx strain, and conversely, (b) by treating the wild-type strain with DNA extracted from a reverted strain. In either cross, erythromycin-resistant transformants should appear having the phenotype of the original (unreverted) mutant strain, if a suppressor mutation is the cause of the phenotypic reversion to wild-type. In (a) the wild-type allele of the suppressor should replace its suppressing homologue in the reverted strain, whereas in (b) the intact erythromycin resistance mutation should be able to be transferred away from its suppressor into the recipient sensitive strain. If the suppressor is linked to the erythromycin resistance mutation, the frequency of erythromycin-resistant transformants would be expected to be lower than in the case where it is unlinked. The spontaneous rate of mutation to erythromycin resistance (10^{-8} to 10^{-9}) sets a lower limit to the frequency of separation of the suppressor from the *ery-r* mutation than would be detectable.

For doubly marked mutants (*ery-r2-r3*), the third and fourth possibilities of explaining reversions may be tested in another way. The reversions that occurred in the *ery-r2-r3* cultures were able to resist 1 μ g. erythromycin per ml., although the parental culture could withstand 40 μ g. erythromycin per ml. If a suppressor arose to counteract one of the mutated sites (*ery-r2* or *ery-r3*), then the level of resistance characteristic of the *ery-r2-r3* strain should be reconstituted by exposure to both the DNA of the normal (i.e. unsuppressed) *ery-r2* strain and the DNA of the normal *ery-r3* strain. For the suppressor should be replaced by its wild-type homologue in either case. On the contrary, if one of the sites had undergone a 'back-mutation', the enhanced level of resistance can be reconstituted only by exposure to one of these DNAs, namely, the DNA bearing the mutation lost in the reverted strain. Eleven independent reversions in *ery-r2-r3* strains were selected. Competent cultures of these reversions were tested by exposing samples separately to DNA *ery-r2 str-r1* and to DNA *ery-r3 str-r1*. Streptomycin resistance transformations were induced readily. However, transformants capable of resisting 5 μ g. erythromycin per ml. were obtained only after exposure to DNA containing the

ery-r3 marker. Although the frequency of transformation to a high level of resistance induced by the *ery-r3* marker was as high as 10^{-4} , the frequency of such transformations by the *ery-r2* marker was less than 10^{-8} . These results permit the conclusion that the *ery-r2* mutation was not involved in these reversions, and that in each case the *ery-r3* mutation underwent a 'back-mutation' to the original wild-type condition.

The results observed in the reversions of the *ery-r2-r3* strains encouraged further analysis of reversions in *ery-r3* and in *ery-r5* strains in order to determine whether suppressor mutations could account for any of these reversions. Five independent reversions in strains bearing the *ery-r5* marker and five independent reversions in strains bearing the *ery-r3* marker were tested by the procedure described in the previous paragraph. Similarly, a reversion in the E21 strain and one in the E24 strain were tested. The DNA used in a given cross always contained, in addition, either the *str-r1* mutation or the *caps*⁺ marker, so that negative findings with respect to the induction of erythromycin resistant transformants were always controlled by positive transfer of some unlinked marker. The results were uniformly negative with respect to the production of erythromycin resistant transformants: such transformants did not appear at a frequency greater than that at which spontaneous mutations arise. Consequently, it may be concluded that most of the reversions in *ery-r3* and *ery-r5* strains are due to back-mutations in the sense just described or to suppressor mutations occurring at sites so closely linked to the original mutated sites that separation by genetic recombination occurs with an undetectably low frequency.

One means exists for distinguishing between the first two possibilities for explaining reversions. If a given erythromycin resistance mutation involved a sufficiently large region of the DNA molecule, and if the reversion resulted in a diminution of the mutated region, and if different reversions caused different non-overlapping portions of the originally mutated site to persist, it may be possible to reconstitute an erythromycin-resistant strain by a 'cross' between two reverted strains. This possibility was examined by means of 'crosses' between a number of reverted strains as shown in Table 9. In no case, however, was resistance to erythromycin recon-

Table 9. Attempts to reconstitute erythromycin resistance by crosses between reverted *ery-r* strains

Host reverted strain	Donor reverted strain	Transformants capable of resisting 0.25 µg. ery./ml.
Rx E21-rev	Rx E24-rev	—
Rx E24-rev	Rx E21-rev	—
Rx <i>ery-r5</i> -rev no. 2	Rx <i>ery-r5</i> -rev no. 1	—*
3		—*
4		—*
5		—*
Rx <i>ery-r3</i> -rev no. 1		—*
2		—
3		—
SIH-1 <i>ery-r3</i> -rev no. 1		—*
2		—*
Rx <i>ery-r5</i> -rev no. 1	Rx <i>ery-r3</i> -rev no. 3	—*

* Control: + transfer of *str-r1* or *caps*⁺ marker.

stituted. This finding does not rule out the second possibility of explaining reversions but it does make improbable the assumption of diminution of mutation in a reversion.

DISCUSSION

The molecule of pneumococcal DNA which regulates resistance to erythromycin is apparently distinct from other molecules of pneumococcal DNA which regulate such diverse properties as capsule synthesis, resistance to streptomycin, etc., in the sense that its transfer in transformation reactions is independent of the transfer of the others (Ravin, 1960). This finding is generally interpreted as due to a heterogeneity in the species of DNA molecules contained in a given genome (Hotchkiss, 1951). In any case, it is evident that whatever organelle organizes the DNA content of a bacterium, extraction of the DNA liberates DNA molecules of different kinds into a heterogeneous mixture. It is hoped that future biochemical investigations will reveal the chemical and/or physical differences between these molecules. In addition to the differences between molecules, however, there is evident intramolecular heterogeneity. A molecule affecting a given bacterial function is differentiable into regions having discrete quantitative influences of their own and capable of being separated from each other or brought together by genetic recombination (Ephrussi-Taylor, 1951; Hotchkiss & Evans, 1958; Ravin, 1960; Lacks & Hotchkiss, 1960). The results obtained with the molecule governing erythromycin resistance in pneumococcus supply further evidence in this regard. Current conceptions of the structure of the DNA molecule equate these different regions within the molecule to specific sequences of nucleotides in the linear DNA polymer.

Although they demonstrate the linkage of the five erythromycin resistance mutations, the present experiments are insufficient to determine the precise locations of these mutations with respect to each other. Further investigations are currently in progress with the aim of clarifying their spatial relationship. Nevertheless, the work reported at this time describes certain anomalous situations which appear to be of considerable interest for our understanding of intramolecular recombination (Ravin, 1961). These situations include the following:

(1) The frequency of recombination between given sites on the endogenous (host) and transforming (donor) DNA molecules appears to vary according to the genetic composition of the recombining molecules. When, for example, the sensitive parental strain *ery-s2-s3-s5* is exposed to the triply-marked DNA from the recombinant *ery-r2-r3-r5*, the most frequent classes of transformants produced are *ery-r2-s3-r5*, *ery-s2-r3-r5* and *ery-r2-r3-r5*. In other words, the *ery-r5* mutation tends to be transferred along with one or both of the other mutations. Such a result would be readily explained on the grounds that the *ery-r5* mutation lies between the *ery-r2* and *ery-r3* mutations, so that a double 'cross-over' within a limited segment of the DNA molecule would be necessary to free *ery-r5* from the mutations on either side of it. Such a double 'cross-over' may be expected to occur infrequently. On this hypothesis, when the *ery-s2-s3-r5* mutant is treated with DNA from the recombinant *ery-r2-r3-s5*, the transformant *ery-r2-r3-s5* should be produced more frequently than the transformant *ery-r2-r3-r5* which would require the same type of double 'cross-over' as has just been invoked. On the contrary, however, the *ery-r2-r3-r5* recombinant is the more frequent product in this cross. Similarly, to take another example, when *ery-r3* is crossed with *ery-r6*, the recombinant *ery-r3-r6* is rare. On the other hand, when *ery-s3-s6* is treated with DNA *ery-r3-r6*, the frequency of the

recombinant *ery-s3-r6* is relatively high. Yet in all respects, except for the specific nature of the sites being confronted in these crosses, the recombinations involved are similar. These results are probably similar to those of Lacks & Hotchkiss (1960), who obtained quite different recombination frequencies in reciprocal crosses between 'maltase' mutants in pneumococcus. It is possible that all of these anomalous results in transformation-mediated recombinations may be eventually explicable by a model, like that discussed by Ephrussi-Taylor (1961), in which the relative lengths of the mutated sites, as well as the distances between them, influence the frequency of recombination.

(2) When the sensitive *ery-s2-s3-s6* strain is treated either with DNA from the recombinant *ery-r2-s3-r6* or with DNA from the recombinant *ery-s2-r3-r6*, the transfer of the two resistance mutations occurs more frequently than the transfer of a specific single mutation. In the case of DNA *ery-r2-s3-r6*, it is *ery-r6* that is transferred rarely relative to the transfer of *ery-r2-r6*. In the case of DNA *ery-s2-r3-r6*, it is *ery-r3* that is transferred rarely relative to the transfer of *ery-r3-r6*. At the present time, it is premature to formulate a model to account for these results. It is interesting, nevertheless, that these two cases provide exceptions to the general rule that a linked pair of mutations is transferred less frequently than the individual mutations (for review, see Ravin, 1961).

Obviously, more crosses need to be conducted using all possible combinations of donor and recipient genotypes. Such experiments are in progress. The results of these further studies should throw light, not only upon the unusual situations described above, but also on the order of the mutated sites in the molecule of DNA governing resistance to erythromycin. It is worth remarking, however, that all five independent spontaneous mutations to erythromycin resistance have been shown to be transferred by the same molecule of DNA. This finding is in support of the possibility, outlined by Bryan (1961), that the different genetic factors known to underlie multi-step resistance to antibiotics are not unlinked polygenes, but rather closely linked members of a complex locus. The action of the latter, as in Bryan's studies on streptomycin resistance, may be affected by modifier genes, which do not confer any resistance by themselves.

Many previous genetic studies of micro-organisms and viruses have indicated the occurrence of what we have referred to above as 'back-mutations', i.e. reverse-mutations occurring at or extremely close to the original sites of mutation. Thus, it is not surprising to find further evidence of such alterations with the genetic material of pneumococci. Since, however, individual DNA molecules are the vectors of genetic transfer in transformation reactions, one has the unique opportunity of localizing the site of the 'back-mutation', not only in terms of genetic (recombination) distances, but also in particular regions of specific DNA molecules. One knows at least, from the present studies, that a 'back-mutation' may occur on the same molecule of DNA as that of the original mutation, and, indeed, may occur so close to the site of the latter as to be inseparable from it by genetic recombination. The question as to whether a 'back-mutation' always restores the original configuration of the genetic material is an interesting one, and is open to analysis. It may be possible to find at some time that a cross between two reversions restores a mutant condition, although our preliminary attempts in this regard have been uniformly negative. If such a positive case were found, evidence would be provided for the existence of different 'wild-type' states of genetic material.

The phenotypic interactions of the different mutations probably reflect the

functional differentiation of the genetic locus concerned with erythromycin resistance. It has been noted that two different mutations of the *ery* locus often interact to produce a more-than-additive, or synergistic, enhancement of resistance to erythromycin. However, this is not always the case. The *ery-r5* mutation does not add appreciably to the level of resistance of a strain already bearing the *ery-r2* or *ery-r3* mutation. Moreover, it antagonizes the action of the *ery-r2-r3* duplex. Finally, the *ery-r7* mutation does not cause any synergistic effect when coupled with either *ery-r6* or *ery-r5*. While it is altogether possible that *ery-r7* is epistatic to *ery-r6* and *ery-r5*, this cannot be readily determined from the present data since the sum of the effects of *ery-r7* and *ery-r5* (which would confer a maximum level of resistance of 11 μ g. erythromycin per ml.) is indistinguishable from an epistatic effect of *ery-r7* (which would confer a maximum level of resistance of 10 μ g. per ml.). New experiments are being planned to test the possible epistasis of the *ery-r7* mutation. It appears likely, in any event, that *ery-r7* acts physiologically in a different manner from that of the other mutations, because of its unique rate of phenotypic expression. Unfortunately, as yet little is known of the biochemical mechanism of erythromycin resistance. No enzymic activity capable of destroying erythromycin has been found in cultures of several different species of erythromycin resistant bacteria (Haight & Finland, 1952a). On the other hand, the inhibitory action of erythromycin against the diphtheria bacillus is known to be antagonized by pantothenic acid, β -alanine and L-carnosine (Brown & Emerson, 1953). This finding is suggestive of a specific metabolic block produced by the antibiotic and furnishes a means of determining the mode of resistance to erythromycin. With such information in hand, it would be of considerable interest to relate the observed epistatic, antagonistic and synergistic interactions of the erythromycin resistance mutations to the specific biochemical functions they carry out in conferring resistance upon the pneumococcus. In an elegant study by Hotchkiss & Evans (1958), the action of three closely linked mutations conferring resistance to sulphanilamide could be ascribed to specific modifications they imparted to the substrate-binding capacity of an enzyme involved in folic acid synthesis. Such studies provide a powerful means of investigating the relation of genetic fine structure to enzyme structure and activity.

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Transforming Activities and Base Contents of Deoxyribonucleate Preparations from Various *Neisseriae*

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SUMMARY

Genetic transformation was investigated among *Neisseria* spp. whose normal habitat is the nasopharynx of humans. Seven species, as characterized in *Bergey's Manual* (1957), were represented. Deoxyribonucleate (DNA) preparations from streptomycin-resistant mutants of *N. meningitidis*, *N. perflava*, *N. flava*, *N. subflava*, *N. sicca*, and *N. flavescens* conferred resistance upon streptomycin-susceptible parent strains of the corresponding species (intraspecific transformation) and of each other species (interspecific transformation). Ratios of interspecific to intraspecific transformation were 0.01 or higher for all possible combinations of DNA and recipient cells of the six species. On the other hand, *N. catarrhalis* cells, which exhibited high frequencies of intraspecific transformation, were not transformed at detectable frequencies by DNA from any of the six *Neisseria* species listed above. In turn, DNA from *N. catarrhalis* had little or no transforming activity for these other *neisseriae*.

Possible evidence of structural differences between these DNA's was sought by analysing the base contents of transforming preparations. The bases adenine, thymine, guanine and cytosine were present in about equal proportions in the DNA's of the six *Neisseria*: *meningitidis*, *perflava*, *flava*, *subflava*, *sicca* and *flavescens*. In DNA preparations from two strains of *N. catarrhalis*, however, adenine and thymine predominated. The ratio (adenine + thymine/guanine + cytosine) was higher than 1.4 compared to 1.0 for the others.

INTRODUCTION

Neisseria meningitidis undergoes genetic change (transformation) affecting capsular antigen specificities (Alexander & Redman, 1953) or response to streptomycin (Catlin, 1960*a*) following brief exposure to solutions of deoxyribonucleate (DNA). The customary source of DNA is experimentally lysed cells. However, genetically active DNA may be found also in the extracellular environment of *N. meningitidis* (Catlin, 1960*a*) and *N. sicca* (Catlin, 1960*b*), presumably owing to spontaneous cellular lysis of some proportion of the populations. DNA can be isolated from the supernatant fluids of centrifuged broth cultures and purified. Moreover, the crude DNA-containing culture slimes themselves elicit transformation. Both *N. sicca* and *N. meningitidis* culture slimes have transforming activity for *N. meningitidis* cells (Catlin, 1960*a, b*).

Extracellular transforming activity has been demonstrated also in cultures of pneumococcus (Ottolenghi & Hotchkiss, 1960). These findings taken together with the knowledge that transformation can occur *in vivo* (see Austrian, 1952) give substance to the idea that transformation does occur in nature, and that it may be a regular method of genetic transfer for some bacteria. Some of the consequences for bacteria of genetic recombination have been discussed by Ravin (1960).

A variety of *Neisseria* spp. inhabit the nasopharyngeal mucosa of man. They may be numerous and proximate in this niche, conditions which should have provided opportunities for recombination, if it occurs. This group appeared favourable, therefore, for investigations of genetic transfer. One or more representatives of each of seven *Neisseria* species (as characterized in *Bergey's Manual*, 1957) which inhabit the nasopharynx were examined quantitatively for their capacity to become transformed to streptomycin resistance following exposure to preparations of DNA isolated from streptomycin-resistant mutant strains derived from each of the seven species. In addition, base analyses were carried out on transforming preparations in an attempt to correlate DNA composition with affinities among the *Neisseria* inferred from the transformation tests; preliminary reports have appeared (Catlin, 1960*c*, 1961).

METHODS

Media. Heart infusion broth (Difco) with or without 0.3% (w/v) yeast extract (Difco) was supplemented after sterilization with 250 µg. ribonucleic acid (Nutritional Biochemicals Corporation)/ml., 0.00005 M-sodium glutamate and 0.0005 M-calcium chloride added separately as sterile solutions (Catlin, 1960*a*). In these media, designated HIY-1 or HI-1, luxuriant growth of all strains was obtained in shaken cultures. HIY-1 agar was used in all plating procedures. Concentrations of agar (Difco) employed were 1.4% (w/v) (hard agar) or 0.7% (w/v) (soft agar).

To eliminate aberrant responses characteristic of surface growth on dry media, agar was freshly poured on the day it was to be streaked. Hard agar bottom layers for assays were dispensed (in volumes of 20 ml. \pm 0.5 ml.) 3–5 days before use, and plates were held at room temperature; when overlaid with inoculated soft agar, surface moisture was negligible.

Capacity to produce acid from carbohydrates was examined by using HIY-1 medium with 1% (w/v) agar, phenol red 0.015 mg./ml., and 0.5% (w/v) of either glucose, maltose, fructose, sucrose, mannitol or lactose. For the latter additions filter-sterilized 20% (w/v) solutions were added aseptically. Media were tubed with a butt and a short slope, and were inoculated by stabbing the deep agar and streaking the surface.

Characterization of strains. Table 1 lists the *Neisseria* strains investigated, their sources and designations. Strain numbers preceded by Ne were from the culture collection of the department. All strains were Gram-negative cocci, commonly arranged in pairs with adjacent sides flattened. They grew well on HIY-1 agar, and were oxidase- and catalase-positive. Chromogenesis, if any, was essentially characteristic of the species, as given in *Bergey's Manual* (1957).

None of the strains produced acid from mannitol or lactose; the (control) medium lacking carbohydrate was invariably alkaline. Reactions of the strains in maltose, fructose and sucrose corresponded, with one exception, to descriptions in *Bergey's Manual* (1957). The exception related to reactions of *Neisseria subflava* strain 11076.

The 'species' is characterized as producing acid from glucose and maltose only. Acid was produced from maltose and fructose within 1-2 days, and usually from sucrose after 4-6 days of incubation. Reactions of this strain, as well as strains of several other species (e.g. *N. sicca* Ne 12 and *N. flava* 4), were irregular in the glucose-containing medium; acid was either not produced or was produced after a delay, as previously reported (Pelczar & Doetsch, 1949; Topley and Wilson's *Principles*, 1955).

All strains were highly susceptible to the antibacterial action of crystalline dihydrostreptomycin sulphate (Squibb). This antibiotic will be referred to as DST, and strains resistant to its action as streptomycin-resistant (*str-r*). Spontaneous single-step mutations conferring resistance to at least 500 µg. DST/ml. were obtained from all seven species, essentially as described (Catlin, 1960*a*).

Table 1. *Sources of Neisseria strains*

Designation		Source	Year isolated (i) or received (r)
Strain	Species		
Ne 15	<i>N. meningitidis</i>	Spinal fluid, fatal, non-epidemic meningitis	1955 (i)
Ne 16	<i>N. perflava</i>	Throat culture, outpatient	1957 (i)
Ne 20	<i>N. perflava</i>	Throat culture, outpatient	1958 (i)
4	<i>N. flava</i>	University of Maryland Collection*	1960 (r)
55	<i>N. flava</i>	University of Maryland Collection*	1960 (r)
JJ IIA	<i>N. flava</i>	Statens Seruminstitut Collection, Copenhagen†	1960 (r)
2104	<i>N. flava</i>	Sputum culture, Walter Reed Army Institute of Research, Washington	1960 (i)
2105	<i>N. flava</i>	Sputum culture, Walter Reed Army Institute of Research, Washington	1960 (i)
11076	<i>N. subflava</i>	American Type Culture Collection	1960 (r)
Ne 12	<i>N. sicca</i>	Throat culture, healthy student	1954 (i)
13120	<i>N. flavescens</i>	American Type Culture Collection, N.I.H. strain N 155*‡	1960 (r)
Ne 11	<i>N. catarrhalis</i>	University of Rochester Collection	1954 (r)
Ne 13	<i>N. catarrhalis</i>	New York State Department of Health, strain 34105	1954 (r)

* = strain examined by Hajek, Pelczar & Faber (1950); † = strain examined by Jessen (1934); ‡ = strain examined by Branham (1930).

Organisms were stored at -60°. A number of similar suspensions of each strain was frozen at one time for subsequent use.

DNA preparations. Streptomycin-resistant strains were subcultured several times on HIY-1 agar supplemented with 500 µg. DST/ml. The last subculture served as inoculum for antibiotic-free HIY-1 broth cultures (incubated at 37° on a shaker) or HIY-1 agar (2%, w/v) plates (incubated at 37° with 70% humidity). Cultures were harvested after 18-22 hr. Organisms were lysed with sodium dodecylsulphate, as described (Catlin, 1960*a*). The DNA-containing fibrous masses obtained by precipitation with 2 volumes of ethanol were purified by methods (Catlin & Cunningham, 1958; Catlin, 1960*a*) which included two separate steps of deproteinization with sodium dodecylsulphate; each was followed by a step involving centrifugation of the DNA solution (in M-NaCl) for 110 min. (32,000*g*; 3°), and precipitation of DNA fibres with ethanol. The final DNA solutions (in 0.14M-NaCl) were prepared using aseptic

precautions (Catlin, 1960*a*), and were found to be sterile. Concentration of DNA was determined by the diphenylamine reaction (Dische, 1955).

Transformation tests. A procedure developed during quantitative transformation studies of *Neisseria meningitidis* (Catlin, 1960*a*) was applied uniformly to all *Neisseria* species. It involved the following steps.

(1) Preparation of suspension of recipient cells. Punctiform surface colonies were picked from HIY-1 agar which had been incubated 11.5–13 hr. in a water-jacketed incubator at 37°. A homogeneous suspension of cells was prepared in warm (36°) HI-1 broth. Occasionally the suspension was coarse, in spite of vigorous repeated expulsion from a pipette; it was centrifuged briefly, and the supernatant fluid was used. Suspensions having a barely visible turbidity contained about 10^7 colony-forming units/ml. A further dilution was made in warm broth to give the desired population size.

Absence of cellular aggregation, a condition rarely attained with cocci, is essential for an accurate determination of transformation frequencies. Among the *Neisseria* spp. examined, *N. meningitidis* was exceptional in providing suspensions having usually about 95% single or paired cocci (Catlin, 1960*a*). Other species gave suspensions having 5–30% of aggregates containing 3–8 cells, most of which numbered 3–4. (Strains of *N. sicca* and *N. catarrhalis*, which were most troublesome, produced no fewer aggregates in shaken HIY-1 broth cultures). As the same cellular suspension was used for comparisons of different transforming preparations, results were affected equally by aggregation, however, and ratios of interspecific to intraspecific transformation were found to be reproducible.

(2) Exposure to DNA. As soon as the cellular suspension was prepared, 1.5 ml. was added to 1.5 ml. of each DNA preparation (20.0 μ g./ml. diluted in HI-1 broth). Each reaction mixture (in 25 \times 150 mm. screw-cup tube) was incubated at 36° for 30 min., whereupon 0.03 ml. of a solution of sterile pancreatic deoxyribonuclease (Worthington) 1 mg./ml. with magnesium, was added to destroy unbound transforming DNA.

(3) Assay of number of 'cells'/ml. exposed = *E*. One or more test populations was sampled just after addition of deoxyribonuclease. Measured volumes of appropriate dilutions in HI broth were added to tubes containing 4 ml. of HIY-1 soft agar (liquefied and held at 44°). These were poured over supporting layers of HIY-1 hard agar. Plates were incubated at 37° for 2–3 days. The value *E* was calculated from the mean number of colonies on five plates, which usually agreed within 10%.

(4) Assay of number of transformants/ml. = *T*. All reaction mixtures were assayed within 90 min. of initiation of exposure. A sample of (0.1–2.0 ml.) was mixed with 40 ml. of HIY-1 soft agar (at 44°); immediately the entire volume was dispensed in 4 ml. aliquots on the surfaces of a set of 10 HIY-1 hard agar plates. Five min. later the plates were placed without stacking at 37°. Five hr. after the time of initial exposure of cells to DNA, plates were removed to room temperature, and each was overlaid with a 4-ml. top layer of HIY-1 soft agar containing DST in a quantity sufficient to give 500 μ g. DST/ml. after diffusion of the antibiotic through the underlying agar. Plates were left at room temperature for a further 45 min.; then they were returned to 37° and were not stacked until each had warmed uniformly. Colonies were counted after incubation at 37° for 3–4 days. The oxidase reaction was used to check all colonies on plates having fewer than ten colonies.

A control reaction mixture containing transforming DNA which had been inactivated by addition of deoxyribonuclease 5 min. before adding recipient cells was included in all transformation tests (Catlin, 1960*a*). Streptomycin-resistant (mutant) colonies were very rarely found.

Analysis of base contents of transforming DNA preparations. RNA was removed as described by Smith & Wyatt (1951). After drying from acetone, DNA was hydrolysed as described by Wyatt & Cohen (1953), using test tubes called ignition tubes in the U.S.A. (There is danger of the tubes exploding during or after heating.) Bases were then determined as described by Wyatt (1951) except that the differential extinction method (Vischer & Chargaff, 1948) was used, the coefficients being given by Bendich (1957).

Table 2. Transformation of *neisseriae* by DNA preparations (final concentration 10 µg. DNA/ml.) from streptomycin-resistant strains of the same species

Streptomycin-susceptible recipients			Derivation of DNA donor	<i>Str-r</i> transformants No./ml. (T)	$\frac{T}{E} \times 10^5$
Species	Strain	No. exposed/ml. (E)			
<i>N. meningitidis</i>	Ne 15	4.6×10^6	Ne 15	15,700*	341.8
<i>N. perflava</i>	Ne 16	1.1×10^6	Ne 16	17,390*	1,580.9
	Ne 16	1.1×10^6	Ne 20	12,080	1,098.2
	Ne 20	1.4×10^6	Ne 16	313	22.4
	Ne 20	1.4×10^6	Ne 20	941	67.2
	4	1.7×10^6	4	17,860	1,050.6
<i>N. flava</i>	4	1.7×10^6	2104	16,070	945.3
	4	1.7×10^6	2105	13,260	780.0
	4	1.7×10^6	JJ IIA	7,470	439.4
	4	3.4×10^6	4	10,260*	301.8
	11076	6.5×10^6	11076	78*	1.2
<i>N. subflava</i>	Ne 12	3.0×10^6	Ne 12	199*	6.6
<i>N. sicca</i>	13120	1.1×10^7	13120	256*	2.3
<i>N. flavescens</i>	Ne 11	8.4×10^4	Ne 11	12,140*	14,452.4
<i>N. catarrhalis</i>	Ne 11	8.4×10^4	Ne 13	6,610	7,869.0
	Ne 13	2.0×10^6	Ne 11	95	4.8
	Ne 13	2.0×10^6	Ne 13	330	16.5

* Data used in connexion with Table 3.

RESULTS

Intraspecific transformation. Representatives of all seven species of *Neisseria* examined were transformed from streptomycin-susceptibility to streptomycin-resistance by DNA extracted from streptomycin-resistant strains of the corresponding species. Table 2 shows transformation ratios obtained for various strains. Ratios were consistently low for the single strains of *N. subflava*, *N. sicca*, and *N. flavescens* examined; between 1 and 10 transformants/100,000 treated cells (colony-forming units) were obtained in repeated tests. In comparison, high transformation ratios were regularly obtained with certain strains of the other four species. Transformation of *N. catarrhalis* strain Ne 11 varied in five independent experiments from about 5 to 15 % of the treated population. In one of these tests assays of both transformants (T) and total exposed cells (E) were carried out on fifteen plates containing an identical inoculum; ten plates were overlaid with DST-containing medium for determination of T, and five plates without overlays were used for

counts of total colonies (E). Cellular aggregation presumably gave an upward bias to T/E values, and contributed to differences between the values found in independent tests of the same recipient. One of the most extreme examples of such a difference is shown in Table 2, *N. flava* 4.

Various strains of the same species differed considerably in capacity to become transformed, as shown by T/E values for *Neisseria perflava* and *N. catarrhalis* strains (Table 2). Even greater differences were exhibited by the five *N. flava* strains (Table 1). Only strain 4 was transformed at a useful frequency. No transformants were detected in single exploratory tests of strains 55 and JJ IIA (T/E $\times 10^5$ ratios were less than 0.007 and 0.06, respectively). Though a few transformants were obtained with strains 2104 and 2105, ratios were very low (0.025 and 0.017). However, DNA preparations obtained from four of these strains (DNA was not prepared from strain 55) were active in eliciting transformation of strain 4 cells (Table 2).

Interspecific transformation. Table 3 and those portions of Table 2 marked by an asterisk give results of seven representative experiments, each of which tested recipient cells of a given species with transforming DNA from all seven species. Data marked by an asterisk in Table 2 are numbers of transformants/ml. obtained in tests of each recipient with homologous DNA; these intraspecific transformation values were compared with the number of transformants/ml. elicited by heterologous DNA preparations (interspecific transformation) in the same experiment, and results are expressed in Table 3 as ratios of interspecific to intraspecific transformation.

Table 3. Ratios of interspecific to intraspecific transformation among *Neisseria* spp.

Recipient cells	DNA preparations from streptomycin-resistant strains of						
	<i>N. meningitidis</i> (Ne 15)	<i>N. perflava</i> (Ne 16)	<i>N. flava</i> (4)	<i>N. subflava</i> (11076)	<i>N. sicca</i> (Ne 12)	<i>N. flavescens</i> (13120)	<i>N. catarrhalis</i> (Ne 11)
<i>N. meningitidis</i> (Ne 15)	*	0.091	0.054	0.022	0.036	0.047	<0.0000
<i>N. perflava</i> (Ne 16)	0.119	*	0.655	0.192	0.141	0.064	<0.0000
<i>N. flava</i> (4)	0.312	0.785	*	0.188	0.342	0.142	<0.0000
<i>N. subflava</i> (11076)	0.577	4.680	3.949	*	0.666	1.154	0.006
<i>N. sicca</i> (Ne 12)	0.020	0.025	0.010	0.010	*	0.020	0.005
<i>N. flavescens</i> (13120)	0.180	0.262	0.094	0.070	0.051	*	<0.002
<i>N. catarrhalis</i> (Ne 11)	<0.000002§	<0.000002§	<0.000002§	<0.000002§	<0.000002§	<0.000002§	*

* = 1.0, intraspecific transformation; see corresponding data of Table 2; † = 1 streptomycin-resistant colony/2 ml. sample; ‡ = 1 streptomycin-resistant colony/ml. sample; § = no transformants found in samples of 3×10^6 colony-forming units/ml., 14 % transformants being elicited by homologous DNA in the same experiment (Table 2, Ne 11).

Ratios (Table 3) were 0.01 or higher for all possible combinations of recipient cells and transforming DNA involving the six *Neisseria* species: *meningitidis*, *perflava*, *flava*, *subflava*, *sicca*, and *flavescens*; each recipient was examined in three or more independent experiments. The interspecific transformation values for *N. sicca* were based on colony counts ranging from 1 to 5/ml. (as compared with 199/ml. for intra-

specific transformation (Table 2). Accordingly, their reliability is doubtful, although very similar results were obtained in two other experiments with strain Ne 12.

Transformation was not detected following treatment of *Neisseria meningitidis*, *N. perflava* (strains Ne 16 and Ne 20), *N. flava*, or *N. flavescens* recipients with DNA preparations from *N. catarrhalis*. Whether the rare streptomycin-resistant colonies found in tests of *N. subflava* and *N. sicca* represented transformants or spontaneous mutants cannot be determined from available data. One of four tests of *N. subflava* and two of three tests of *N. sicca* showed between one and three streptomycin-resistant colonies/2 ml., where corresponding numbers of cells treated with depolymerized DNA (controls) gave no resistant colonies. It is hoped that genetic tests of strains originating from such colonies will indicate their origin; we are at present inclined to regard them as spontaneous mutants, reversing the view expressed in a preliminary report (Catlin, 1960c).

Recipient cells of *Neisseria catarrhalis* strains Ne 11 and Ne 13, examined in five independent experiments, did not undergo interspecific transformation. In one experiment (Ne 11, Table 2) 12,140 transformants/ml. were elicited by treatment with *N. catarrhalis* DNA of a population of 8.4×10^4 colony-forming units/ml.; in contrast, a higher concentration of the same cellular suspension (3.0×10^6 colony-forming units/ml.) gave no transformants following exposure to DNA preparations from each of the other six species (ratios of interspecific to intraspecific transformation less than 0.000002 (Table 3).

Base composition of DNAs. The base contents of transforming DNA preparations are given in Table 4. The ratio (adenine + thymine/guanine + cytosine) for *Neisseria meningitidis* DNA is in agreement with the value (1.00) found by Lee, Wahl & Barbu (1956) for another strain of the same species.

Table 4. *Purine and pyrimidine contents of DNA preparations obtained from streptomycin-resistant strains of Neisseria spp.*

Derivation of DNA donor	Mole/100 mole total bases				$\frac{A+T}{G+C}$
	Guanine	Cytosine	Adenine	Thymine	
<i>N. meningitidis</i> (Ne 15)	25.7	25.6	23.5	25.2	0.95
<i>N. perflava</i> (Ne 16)	25.4	24.9	26.0	23.8	0.99
<i>N. perflava</i> (Ne 20)	25.4	24.4	25.2	24.9	1.01
<i>N. flava</i> (JJ IIA)	25.0	24.5	25.5	25.0	1.02
<i>N. subflava</i> (11076)	24.1	26.4	21.9	27.6	0.98
<i>N. sicca</i> (Ne 12)	26.4	25.1	24.0	24.4	0.94
<i>N. flavescens</i> (13120)	25.6	24.5	23.6	26.2	0.99
<i>N. catarrhalis</i> (Ne 11)	19.9	20.8	28.6	30.6	1.45
<i>N. catarrhalis</i> (Ne 13)	19.1	21.0	27.9	32.1	1.50
<i>N. catarrhalis</i> (Ne 13 (11 str-r))*	19.9	21.4	28.2	30.5	1.42

* Streptomycin-resistant strain derived from Ne 13 by transformation with DNA from Ne 11 str-r.

DISCUSSION

The near-identity of the base ratios of DNA preparations from six different *Neisseria* spp.: *meningitidis*, *perflava*, *flava*, *subflava*, *sicca*, and *flavescens* (Table 4) is in accordance with expectation based on the results of transformation experiments. These DNA preparations elicited genetic change of recipient cells in all

thirty-six possible combinations involving the six kinds of *Neisseria* (Table 3). Thus, representatives of these six species (as defined in *Bergey's Manual*, 1957) may be regarded as members of a single group (group 1) in so far as they are related by the possibility of genetic transfer. On the other hand, a barrier to the transfer of genetic information appears to exist between group 1 strains and strains of *N. catarrhalis*. Recipient cells of the latter were not transformed by DNA preparations from group 1 strains, and conversely, DNA from *N. catarrhalis* had little or no transforming activity for recipient cells of group 1. The significant difference between the DNA base ratios of members of group 1 on the one hand, and *N. catarrhalis* on the other, indicates a possible structural basis for the barrier between the two groups.

A growing body of information suggests that genetic transfer occurs only between bacteria having DNA with similar base ratios (see discussions of this subject by Lanni, 1960, and by Ravin, 1960). Base ratios may range at least from 0.4 to 2.7, as shown by an investigation of the DNA of 60 strains of true bacteria (Lee *et al.* 1956). A significant difference between ratios found for any two strains implies remoteness of relationship; accordingly, transfer of genetic information between the two would not be expected. Although there is no agreement concerning the scope of the bacterial genus, the inclusion of *catarrhalis* strains in the genus *Neisseria* appears illogical from the evolutionary point of view.

In general, where adequate quantitative experimental design has eliminated bias due to differential selection of either the transformed or untransformed elements of the bacterial population, higher frequencies are obtained in intraspecific than in interspecific transformation tests (Schaeffer, 1958; but see Bracco, Krauss, Roe & MacLeod, 1957). Leidy, Hahn & Alexander (1956, 1959) have applied an analysis of ratios of interspecific to intraspecific transformation to the taxonomy of the genus *Haemophilus* on the premise that such ratios reflect the degree of relationship of donor and recipient cells. Thus far, however, quantitative investigations of interspecific transformations have been restricted to streptomycin resistance; in view of findings by Green (1959) interpretation of interspecific transformation data involving this single characteristic should be made with caution. He showed that heterogeneity of recipient pneumococcus strains may have an unequal influence on transformation frequencies determined for two different characteristics. The frequency of transformation to streptomycin resistance was consistently lower with one recipient than with a second, whereas these two pneumococcal strains exhibited equal frequencies of transformation to erythromycin resistance following exposure to a single doubly-marked transforming preparation.

The status of several species of *Neisseria* described in *Bergey's Manual* (1957) is doubtful. The Subcommittee on the family Neisseriaceae (1954) recognized the need to clarify the classification of all members of the Neisseriaceae. Topley and Wilson's *Principles* (1955) prefers to recognize as separate species among the Gram-negative cocci of the human nasopharynx only *N. meningitidis*, *N. flavescens* and *N. catarrhalis*; the remaining types, with the possible exception of *N. sicca*, would be combined into a single species, *N. pharyngis*. This suggestion that *N. perflava*, *N. flava*, and *N. subflava* are not sufficiently distinct to warrant separate species designations is in harmony with the present evidence from transformation tests.

DNA preparations from strains of *Neisseria flava* isolated in areas of the world as distant as the United States and Denmark (Table 1) and at times separated by

more than 25 years were capable of conveying genetic information to *N. flava* strain 4 (Table 2). However, the latter was transformed by DNA from strain JJIIIA *str-r* at frequencies lower than those elicited in the same experiment by DNA preparations from *N. perflava* (both strains Ne 16 *str-r* and Ne 20 *str-r*).

The response of *Neisseria subflava* strain 11076 was unique among the *Neisseria* spp. investigated. Recipient cells were transformed at higher frequencies by DNA preparations from streptomycin-resistant strains of *N. perflava* and *N. flava* than by DNA from *N. subflava str-r*. Further study may show that the response of this strain to homologous DNA is an example of 'depressed' transfer of the streptomycin resistance marker (Green, 1959). The atypical fermentation reactions (see 'Methods') suggest that this strain is not a representative of *N. subflava*. Strains which do correspond to the description of *N. subflava* (as given in *Bergey's Manual*, 1957) are rarely encountered (report of the Subcommittee on the family Neisseriaceae, 1954). A genetic study of available strains, together with further investigations of strain 11076 and representatives of *N. flava* and *N. perflava*, may clarify their taxonomic relations. Further discussion of possible taxonomic implications of ratios of inter-specific to intraspecific transformation is better postponed until results of tests with other markers are available.

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The Role of Magnesium Ions in the Growth of *Salmonella* Phage Anti-R

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SUMMARY

Magnesium or certain other divalent metal ions are needed for the adsorption of phage anti-R to its host, a 'rough' strain of *Salmonella typhi*. In medium containing magnesium ions the infected organisms burst after a latent period of about 20 min. at 37°, but when infected organisms are diluted into medium without added divalent metal ions phage growth is considerably inhibited. A rapid increase in phage titre occurs when magnesium ions are added late, i.e. at some time after the end of the normal latent period, to dilute cultures of phage-infected organisms. During the interval between infection and this late addition of magnesium ions no appreciable numbers of infective intracellular phage were detected after ultrasonic disruption of the organisms. It is concluded that in addition to being an adsorption cofactor, magnesium functions at some late stage in phage development. Phage anti-R is probably related to Φ X 174; it consists of particles approximately 30 m μ in diameter and the intact phage reacts similarly with formaldehyde.

INTRODUCTION

Many phages require divalent metal ions for growth or fail to act on their specific bacterial hosts in the presence of substances such as sodium citrate and sodium oxalate which bind these ions (see Fildes, Kay & Joklik, 1952). Phage reproduction may be dependent upon divalent metals in a variety of ways. Some phages are unstable in the free state unless metal ions, particularly calcium, are present (Adams, 1949); some are unable to attach to the host in the absence of the ions (Gratia, 1940), and others need the ions to facilitate the entry of phage nucleic acid (Luria & Steiner, 1954). Fildes (1954) established that calcium or magnesium ions are needed for the activity of salmonella phage anti-R (A. 59/6SR Felix). The following paper describes further experiments on the divalent metal requirement of this phage, with special reference to the action of Mg^{++} .

METHOD

Host organism. Phage anti-R is active on 'rough' strains of *Salmonella typhi* and on some strains of *Escherichia coli*. In the work to be described, *S. typhi* R4 derived from NCTC no. 3390 (Kay & Fildes, 1950), was used as the host organism,

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stock cultures being maintained at 4° on slopes of tryptic meat agar and subcultured monthly.

Media. Medium A contained 1% (w/v) peptone (Evans), 7.5×10^{-2} M-NaCl and 2×10^{-3} M-MgSO₄. Medium B was the chemically defined medium of Kay & Fildes (1950) used at half strength. Before use it was supplemented with 2×10^{-2} M-glucose and 0.05% (w/v) decalcified peptone (Fildes, 1954).

Phage. There is some doubt about the precise origin of phage anti-R but it is thought to have been isolated from a patient with paratyphoid A fever. The large size of the plaques formed by the phage suggests that it is very small (Elford & Andrewes, 1932) and this is confirmed by electron micrographs which show that it consists of apparently tailless particles about 30 mμ in diameter.

Phage assay. The standard double-layer plate method was used (Adams, 1950). The layers consisted of medium A solidified with 2% (w/v) agar (Difco) over which a mixture of 2 ml. melted agar medium (medium A containing 1.25% w/v, agar) and 1 ml. host + phage mixture was poured. All phage estimations were done in duplicate. After overnight incubation at 37° plaques of 5 mm. average diameter were formed. This size limited the number of discrete plaques that could be accommodated on a 9 cm. diameter Petri dish and the usual procedure was to incubate the assay plates at 37° for 3 hr. and then to remove them to room temperature (about 20°) overnight. In this way plaques of 2 mm. average diameter, easily discernible against the bacterial background, were formed. There was a linear relation between numbers of plaques and concentration of phage suspension plated up to about 300 plaques/Petri dish.

Preparation of phage suspensions. An initial stock of phage was prepared by three successive single-plaque isolations and inoculation of the last of them into an actively growing culture of *Salmonella typhi* R4 in medium A which was aerated in shaken L-shaped tubes (Monod, Cohen-Bazire & Cohn, 1951) at 37°. When lysis was completed the cell debris was removed by centrifugation and the supernatant fluid decanted into a screw-capped bottle containing a drop of chloroform. Larger volumes of phage suspension were made by inoculating actively growing cultures of *S. typhi* R4 at about 10^8 organisms/ml. in medium A with one-tenth the number of phage. The cultures were aerated vigorously for 3 hr. either in conical flasks attached to a reciprocating mechanical shaker or in rotating flasks (Mitchell, 1949). The cell debris sedimented by centrifugation at 2000 g retained a considerable amount of phage and this could be partially recovered by suspending the debris in 3.3×10^{-2} M-phosphate buffer (10.1 g. Na₂HPO₄. 12H₂O + 0.68 g. KH₂PO₄/l.; pH 7.6) and shaking for a further hour before again centrifuging at 2000 g. The supernatant fluids from both centrifugations were pooled and gave phage titres that varied from 2×10^9 to 1×10^{10} plaque forming units (pfu)/ml. Higher titre phage stocks (up to 2×10^{11} pfu/ml.) were made by alternate cycles of low-speed (2000 g) centrifugation and high-speed (105,000 g) centrifugation in a Spinco model L ultracentrifuge using 3.3×10^{-2} M-phosphate buffer (pH 7.6) containing 0.01% (w/v) serum albumin as the suspending medium.

Measurement of phage adsorption. Fresh cultures of *Salmonella typhi* R4 grown to 10^9 organisms/ml. in medium A at 37° were centrifuged and the bacteria washed once with 3.3×10^{-2} M-phosphate buffer (pH 7.6). Before use the organisms were resuspended in the original culture volume of buffer and further diluted into buffer with or without added divalent metals. After equilibrating 9 ml. bacterial suspen-

sion at 37° for 10 min., 1 ml. phage suspension containing about 10^5 pfu/ml. was added and rapidly mixed. Samples taken at intervals were diluted 10-fold into ice-cold 3.3×10^{-2} M-phosphate buffer (pH 7.6) and quickly assayed for phage before and after centrifugation at 2000 *g* to sediment bacteria and adsorbed phage.

One-step growth experiments. Nine ml. medium B inoculated with 1 ml. of an overnight culture of *Salmonella typhi* R4 in the same medium were aerated in a L-tube at 37° to give about 1.5×10^8 organisms/ml. The culture was concentrated by centrifugation and resuspension of the organisms in 1 ml. of the supernatant fluid. Magnesium sulphate (0.1 ml., 4×10^{-2} M-MgSO₄) and phage anti-R (0.1 ml. of a suspension containing about 2×10^{10} pfu/ml.) were added and the mixture incubated in stationary culture at 37° for 10 min. At the end of this adsorption period 9 ml. ice-cold medium B were added and the organisms sedimented in a cooled centrifuge tube. The bacteria were washed once with 10 ml. ice-cold medium, centrifuged, and after resuspension, diluted into medium B at 37°. The final dilution was aerated in a L-tube at 37° and assayed for phage at intervals. Compared with one-step growth experiments in which the infected culture was not washed, the latent period was not greatly lengthened by this procedure, provided the time between the addition of ice-cold medium and the final dilution into warmed medium was discounted. The method decreased the free phage to less than 1 % of the initial count.

Determination of intracellular phage. The organisms in samples taken during one-step growth experiments were disrupted by one of two methods. The first involved incubation for 90 min. at 37° in a lysing mixture containing 2.5 M-glycine and 10^{-2} M-KCN (Kay, 1952). Preliminary experiments showed that the turbidity of either uninfected or phage anti-R-infected *Salmonella typhi* R4 was decreased more than 85 % after 2-3 hr. incubation in the lysing mixture at 37°, and that the decrease in titre of a phage suspension maintained for 3 hr. in the mixture at 37° was no greater than that of a control suspension in buffer incubated similarly.

The second method used ultrasonic vibration (Anderson & Doermann, 1952). Potassium cyanide to a final concentration of 10^{-2} M was added to prevent further phage growth and the samples were then treated for 5 min. in a Mullard Ultrasonic Generator, Type E 7590 B (Mullard Ltd., London). This period of ultrasonic vibration decreased the viability of a dilute suspension of *Salmonella typhi* R4 to 2 % of the initial count, but periods of ultrasonic treatment as long as 20 min. of dilute phage suspensions did not decrease their titre.

Bacterial counts. The method of Miles & Misra (1938) was used. For routine purposes the number of organisms in a culture was assessed turbidimetrically by using a curve relating counts to the opacities of cultures in medium B in a Hilger 'Spekker' Absorptiometer (Hilger & Watts Ltd., London, N.W. 1).

Spectrophotometry. A Unicam SP 500 Spectrophotometer (Unicam Instruments Ltd., Cambridge) was used with cuvettes of 1 cm. light path.

RESULTS

The stability of phage anti-R

Phage lysates which had been diluted into 3.3×10^{-2} M-phosphate buffer (pH 7.6) and maintained at 37° in unshaken test tubes lost about 20 % of their initial titre in 90 min. This inactivation was not appreciably diminished by the addition to the

diluent of MgSO_4 ($4 \times 10^{-3}\text{M}$), CaCl_2 ($5 \times 10^{-4}\text{M}$) or Na citrate (10^{-2}M). Some of the diluted phage suspensions showed a delayed inactivation which was best seen at an elevated temperature (50°). This delay and the inability to obtain phage titres greater than about 10^{10} pfu/ml. in multistep lysates, in spite of average burst size of 500 in one-step growth experiments in medium A, suggested that some phage may be masked either by aggregation or by reversible inactivation by material present in the lysates. Even after several cycles of alternate low-speed and high-speed centrifugation the concentrated phage suspension still produced a precipitate when incubated with bacterial antiserum, and flagellar material derived from the host bacterium could be seen in electron micrographs of phage prepared in this way. Whenever possible, concentrated phage preparations treated with bacterial antiserum were used for the subsequent experiments, though there was evidence from the occasional variation in the titre of certain stocks that the cause of the masking was not entirely eliminated by antiserum treatment.

*The effect of divalent metal ions on the attachment of phage
anti-R to host bacteria*

There was no adsorption of phage anti-R to washed suspensions of *Salmonella typhi* R4 in $3.3 \times 10^{-2}\text{M}$ -phosphate buffer (pH 7.6) at 37° unless Mg^{++} or other divalent metal ions were present. In the presence of excess bacteria about 90 % of the initial phage input was adsorbed at an exponential rate which was proportional to the concentration of bacteria over the range 2×10^8 to 8×10^8 organisms/ml. The variation of the rate of adsorption of phage with Mg^{++} and other divalent metal ions is shown in Fig. 1. The rates are calculated on the assumption that the adsorption of phage anti-R behaves as a first order reaction (Ellis & Delbrück, 1939).

The effect of divalent metal ions on the stages following attachment

The stage immediately after attachment. The study of the function of divalent metals in the penetration of phage anti-R nucleic acid and in other early stages of the growth cycle was made difficult by the requirement of the metals for adsorption. Puck, Garen & Cline (1951) found with phage T1 that there were two stages in the adsorption to *Escherichia coli* and that the second step was inhibited at low temperatures. With the usual method of measuring phage anti-R adsorption there appeared to be very little attachment at 0° even in the presence of concentrations of Mg^{++} that were effective at 37° . When, however, a comparison is made of the free phage in a host-phage mixture at 0° both before and after dilution it is evident that although attachment occurred at 0° in the presence of Mg^{++} , it was reversible, unlike that at 37° (Table 1).

Advantage was taken of the temperature sensitivity of permanent adsorption to determine whether it, like reversible attachment, is cation dependent. If it is, dilution into medium at 37° of dissociable host-phage complexes that had been formed at 0° should result in the elution of phage unless the medium contains added divalent metal ions. The results of this experiment (Table 2) show that transfer to medium not containing added Mg^{++} but otherwise favourable for phage growth caused bound phage to be released.

Later stages of the phage-growth cycle. If divalent cations function only during the initial stages of the phage-growth cycle their removal once these have been

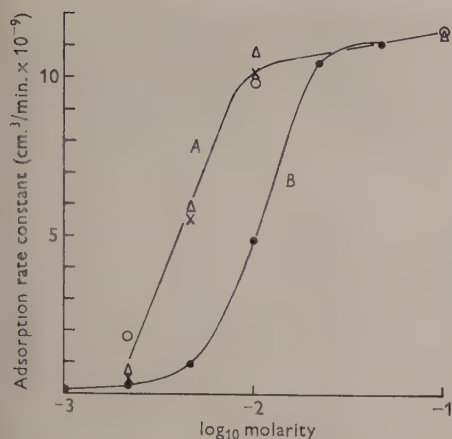


Fig. 1

Fig. 1. Effect of divalent metal ion concentration on the rate of adsorption of phage anti-R to washed suspensions of *Salmonella typhi* R4. Curve A: rates of adsorption in 3.3×10^{-4} M-phosphate buffer (pH 7.6) containing, MgSO_4 , \circ ; CaCl_2 , \triangle ; SrCl_2 , \times . Curve B: rates of adsorption in 3.3×10^{-2} M-phosphate buffer (pH 7.6) containing MgSO_4 , \bullet . Temperature 37° .

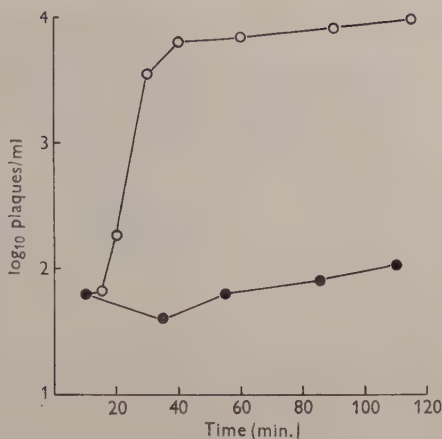


Fig. 2

Fig. 2. Effect of Mg^{++} on the growth of phage anti-R. *Salmonella typhi* R4 was infected with phage anti-R in the presence of 4×10^{-3} M- MgSO_4 . The culture was diluted $1/10^6$ in medium B after 10 min., then finally diluted 10-fold into medium B, without (\bullet — \bullet), and with (\circ — \circ) added MgSO_4 (10^{-3} M). Temperature 37° .

completed should not affect the subsequent development of the phage. In one-step growth experiments the removal of Mg^{++} after as long as 10 min. at 37° had a marked inhibitory effect on the burst size. Compared with the average burst size of about 100 in medium B containing 10^{-3} M- MgSO_4 there was either a decrease or at most a very slight increase in plaque-forming centres when phage anti-R infected organisms were diluted into medium B with no added Mg^{++} (Fig. 2). Under the latter conditions there was in fact an increase in free phage, probably due to the trace amounts of divalent metals in medium B. The transfer of phage-infected bacteria to medium B supplemented with Na citrate (10^{-2} M) caused a decline in the number of plaque forming centres and no increase in free phage.

Table 1. Reversibility of adsorption of phage anti-R at 0°

Phage anti-R was added to a suspension of *Salmonella typhi* R4 in 3.3×10^{-2} M-phosphate buffer (pH 7.6) containing 6×10^{-3} M- MgSO_4 and distributed into two tubes; (A) for incubation at 37° (B) for incubation at 0° . After 3 min. and 15 min., respectively, two samples were removed from both tubes. One sample was centrifuged immediately and the supernatant fluid diluted in buffer before assay; the other was diluted into ice-cold buffer and assayed after centrifugation at the times indicated.

Adsorption tube	Centrifuged before dilution	Centrifuged after dilution		
		0.5 min.	5 min.	10 min.
		unadsorbed phage (%)		
A (37°)	28	39	36	33
B (0°)	46	89	94	99

Table 2. *Effect of Mg^{++} on the second stage of adsorption*

Phage-anti-R was added to a culture of *Salmonella typhi* R4 in medium B containing $4 \times 10^{-3}M$ - $MgSO_4$ at 0° . After 15 min. there was 41% free phage in a sample centrifuged prior to dilution. Two further samples were then diluted 1/200 in medium B with and without added $4 \times 10^{-3}M$ - $MgSO_4$ at 37° . The adsorption was determined at intervals by assay before and after centrifugation.

Time after 1/200 dilution (min.)	No added divalent metal	With $4 \times 10^{-3}M$ - $MgSO_4$
	Unadsorbed phage (%)	
0.25	81	—
0.75	—	46
1.5	90	—
3.0	100	—
3.5	—	42
7.0	—	48
8.5	100	—

The point of action of divalent metals in the post-adsorptive stage

A culture of *Salmonella typhi* R4 in medium B was infected with phage anti-R and after 10 min. was diluted into a L-tube containing medium B supplemented with $10^{-3}M$ - $MgSO_4$. This culture was aerated at 37° and at various times samples were diluted 10-fold into tubes of medium B containing $10^{-3}M$ - $MgSO_4$ and into tubes of medium B containing $10^{-2}M$ -Na citrate. The phage content of the latter series of tubes was determined immediately and both sets of tubes were assayed after 90 min. incubation at 37° . The results (Table 3) show that however late in the growth cycle the infected organisms were transferred to medium deficient in ionized magnesium there was no further increase in phage titre. These results indicated that magnesium ions functioned at some step at the end of the phage development cycle. Further evidence for this came from experiments in which infected bacteria placed in medium B without added Mg^{++} were transferred at various times beyond the normal

Table 3. *Effect of dilution into medium containing Na citrate on the growth of phage anti-R*

A phage-infected culture of *Salmonella typhi* R4 was diluted into medium B containing $10^{-3}M$ - $MgSO_4$ and incubated at 37° . Samples were removed at intervals and diluted 10-fold into tubes of medium B containing $10^{-2}M$ -Na citrate (Series 1) and into tubes of medium B containing $10^{-3}M$ - $MgSO_4$ (Series 2). Series 1 was assayed immediately and both series were assayed after incubation for 90 min. at 37° .

Time after adding phage (min.)	Initial	After 90 min. incubation at 37°	
	Series 1	Series 1	Series 2
	Plaque count		
15	811	71	20,200
18	398	133	20,400
21	1,310	1,130	22,000
26	7,040	7,350	23,700
30	13,700	13,700	20,700
35	18,800	15,600	21,500

end of the latent period (20 min.) to medium B supplemented with divalent metal ions. Figure 3 shows that the late addition of Mg^{++} produces a rapid increase in phage titre. The magnitude of the burst became less the longer the addition of Mg^{++} was delayed (Fig. 4).

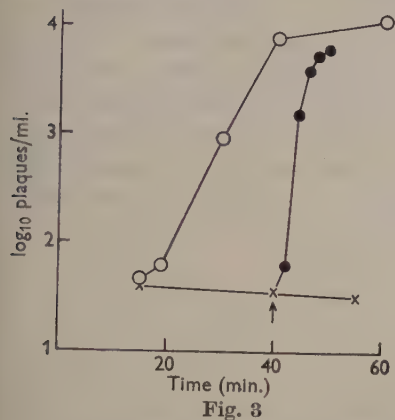


Fig. 3

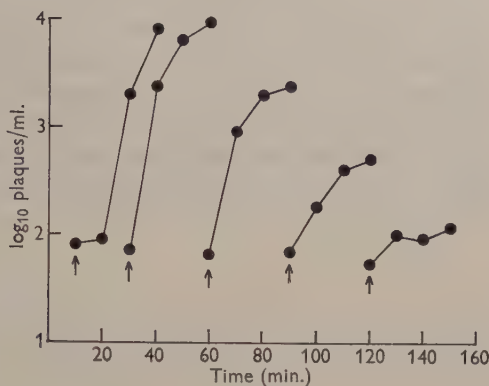


Fig. 4

Fig. 3. Effect of delayed addition of Mg^{++} on the growth of phage anti-R. A culture of *Salmonella typhi* R4 in medium B containing $4 \times 10^{-3} M$ $MgSO_4$ was infected with phage anti-R and then diluted into medium B, with $MgSO_4$, $10^{-3} M$ (O—O); and without added Mg^{++} (x—x). At 40 min. a sample from the latter growth tube was transferred to another tube containing added $MgSO_4$ (final concentration $10^{-3} M$) (●—●). Temperature 37° .

Fig. 4. Effect of delayed addition of Mg^{++} on phage anti-R growth. A culture of *Salmonella typhi* R4 in medium B containing $4 \times 10^{-3} M$ $MgSO_4$ was infected with phage anti-R and diluted into five 1-tubes containing medium B only. At the times indicated by the arrows $MgSO_4$ (final concentration $10^{-3} M$) was added to each of the tubes. Temperature 37° .

The nature of the late metal-dependent step in phage anti-R growth

The only well-recognized stage at the end of the phage-growth cycle is the lytic reaction whereby fully mature phage particles inside the infected host are released into the medium. To ascertain whether or not divalent metals were cofactors for lysis a concentrated culture of *Salmonella typhi* R4 was infected with a 10-fold excess of phage anti-R in the presence of $10^{-3} M$ $MgSO_4$ at 37° . After 10 min. the culture was diluted with cold medium B, centrifuged, washed with cold medium and finally resuspended in the initial volume of medium B without added Mg^{++} . Nine ml. of the suspension were added to two 1-tubes, one of which contained 1 ml. $4 \times 10^{-2} M$ $MgSO_4$ and the other 1 ml. water. The tubes were shaken at 37° and opacity readings were taken at intervals. Phage assays were made at the beginning and at the end of the experiment. The results (Fig. 5) showed that the decrease in turbidity of the cultures was independent of Mg^{++} , though there was no rise in phage titre unless Mg^{++} was present. Similar experiments in which the degree of lysis was determined by the amount of ultraviolet-absorbing material released into the supernatant fluid of infected cultures with or without added Mg^{++} again showed that there was no difference in the degree of cell disruption.

Intracellular growth of phage anti-R

Attempts to show intracellular phage development by glycine + cyanide lysis method (Kay, 1952) were unsuccessful in spite of the good macroscopic lysis pro-

duced when either suspensions of uninfected organisms or those infected with phage anti-R were added to the lysing medium of 2.5 M -glycine + 10^{-2} M -KCN. The failure to detect intracellular phage by this method may be due to chelation of divalent metals by the high concentration of glycine (Albert, 1950). Thus intracellular phage could be shown if samples were added to a mixture of 10^{-2} M -KCN + 10^{-3} M - MgSO_4 but not when added to a mixture of 10^{-2} M -KCN + 10^{-2} M -Na citrate. By using ultrasonic vibration to disrupt phage-infected bacteria which had been diluted into medium B with added Mg^{++} it was possible to show that intracellular phage was formed before the bacteria lysed. In similar experiments in which infected organisms were diluted into medium B without added Mg^{++} there was little increase in the phage content of sonicated or control samples until divalent metal was added (Fig. 6).

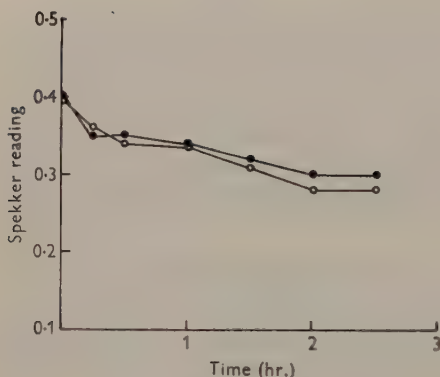


Fig. 5

Fig. 5. Effect of Mg^{++} on the lysis of *Salmonella typhi* R4 by phage anti-R. A culture of *S. typhi* R4 was infected with phage anti-R and after washing was resuspended in medium B, without added Mg^{++} , (\circ — \circ); and with added MgSO_4 (final concentration $4 \times 10^{-3} \text{ M}$), (\bullet — \bullet). Both cultures were aerated at 37° . Initial phage titre = $9 \times 10^6/\text{ml}$. Final phage titre; without added Mg^{++} = $10^6/\text{ml}$.; with Mg^{++} ($4 \times 10^{-3} \text{ M}$) = $2 \times 10^8/\text{ml}$.

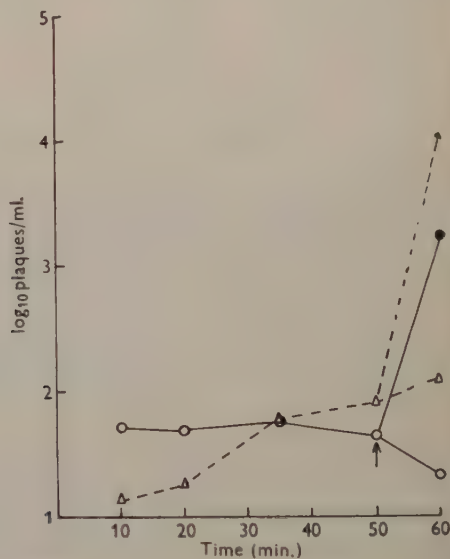


Fig. 6

Fig. 6. Effect of Mg^{++} on the intracellular development of phage anti-R. A culture of *Salmonella typhi* R4 in medium B containing $4 \times 10^{-3} \text{ M}$ - MgSO_4 was infected with phage anti-R and then diluted into medium B without added Mg^{++} . Samples were plated at intervals, before ultrasonic treatment (\circ — \circ) and after 5 min. ultrasonic treatment (\triangle — \triangle). At 50 min. a portion of the culture was transferred to a tube containing MgSO_4 (final concentration 10^{-3} M) and at 60 min. this was plated before ultrasonic treatment (\bullet — \bullet) and after 5 min. ultrasonic treatment (\blacktriangle — \blacktriangle). Temperature 37° .

The relation of phage anti-R to phages S13 and Φ X 174

The small size of phage anti-R particles suggested that the phage might be related to other small phages e.g. S13 and Φ X 174 and like them contain a characteristic type of nucleic acid (Sinsheimer, 1959). Concentrated preparations of phage anti-R were treated with host bacterial antiserum and then taken through a further

cycle of low-speed and high-speed centrifugation. The final suspension of the phage was made in 10^{-2} M-phosphate buffer (pH 7.0) and contained 4.5×10^{11} pfu/ml. The phage was diluted 10-fold into 10^{-2} M-phosphate buffer containing 1.6% (w/v) formaldehyde and the absorption spectrum was measured immediately and after 1, 6 and 23 hr. incubation at 37° . The results (Fig. 7) show that treatment with formaldehyde caused an increase in the extinction in the 260 m μ region, with a shift in the absorption maximum to a greater wavelength. This reaction is one of the characteristics of single-stranded DNA (Sinsheimer, 1959). Other investigations (D. Kay, to be published) have shown that the nucleic acid isolated from phage anti-R has a composition which is indicative of single stranded DNA and that the intact phage differs immunologically from phage S13.

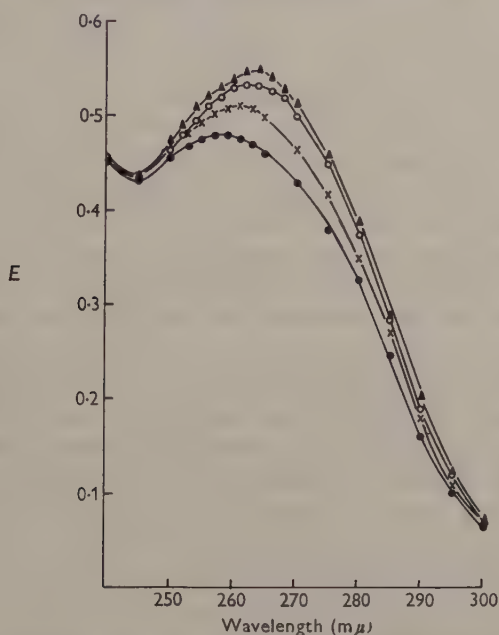


Fig. 7. Effect of formaldehyde on the absorption spectrum of phage anti-R. A suspension of phage anti-R treated with host bacterial antiserum was resuspended in 10^{-2} M-phosphate buffer (pH 7.0) and diluted into the same buffer containing 1.6% (w/v) formaldehyde. Absorption spectrum measured at zero time (●—●); 1 hr. (×—×); 6 hr. (○—○) and 23 hr. (▲—▲). Temperature 37° .

DISCUSSION

The divalent metal requirement for the growth of phage anti-R, a small phage which may contain single-stranded DNA, can be satisfied by magnesium, calcium or strontium ions, and while the effect of Mg^{++} alone has been investigated in detail the other cations must be presumed to act in a manner that is at least qualitatively the same. The promotion of phage anti-R adsorption by Mg^{++} is similar to the action of divalent cations on many other host-phage systems. Puck, Garen & Cline (1951) measured the attachment of phage T1 to *Escherichia coli* B in the presence of different concentrations of alkaline earth metal ions and found that the optimum rate of adsorption was obtained at 5×10^{-4} M irrespective of the metal used. Mag-

nesium, calcium and strontium ions function equally well as cofactors for the adsorption of phage anti-R to suspensions of *Salmonella typhi* R4 in buffer, and the rate of adsorption approaches a maximum at the highest concentration (10^{-1}M) investigated. The fastest rate of adsorption of phage anti-R is considerably greater than the maximum for phage T1 (Puck *et al.* 1951; Garen, 1954) but it is not inconsistent with the small size of the phage anti-R particle.

Adsorption at 0° showed that infection by phage anti-R, like that by phage T1, involves two early reactions, the second of which is temperature-sensitive and leads to the permanent attachment of phage to host bacterium. The need for divalent metal ions for this second step prevented examination of the metal dependence of stages which follow rapidly upon attachment.

Decreasing the concentration of Mg^{++} in the medium for a period as long as 10 min. after adding phage anti-R greatly decreased the burst size and there was evidence for some action of the metal at a late stage of the growth cycle. First, removal of divalent cations by chelation during the rising phase of the burst prevented further increase in the phage titre. Such a result would be expected if Mg^{++} or the other ions acted as cofactors for lysis. This could not be shown by direct measurement of changes in turbidity or by the estimation of ultraviolet-absorbing material released by infected organisms with or without added divalent cations; but it should be pointed out that the experimental conditions were somewhat different from those used previously and did not produce marked clearing even when divalent metals were present. Secondly, the addition of Mg^{++} to infected bacteria in low-metal medium at a time well beyond the end of the normal latent period led to a rapid appearance of infective particles. The speed with which the plaque count increased seems to exclude an action on the multiplication of the phage and the result might also be explained as an action of Mg^{++} on the lytic process. However, the inability to detect appreciable amounts of intracellular phage in organisms in low-metal medium suggests that the metals do function in some process which either leads to phage maturation or is necessary for the maintenance of intracellular phage in an infective state. Rountree (1955) described a calcium-dependent function occurring late during the growth cycle of certain staphylococcal phages. Although the nature of the function was not determined, it appeared in that case to be connected with intracellular stabilization of the phage rather than lysis, since chelation of the metals did not entirely prevent the subsequent liberation of some phage.

I wish to record my appreciation of the encouragement and helpful advice given by Sir Paul Fildes, F.R.S. Part of the work was done during the tenure of a Beit Memorial Fellowship, and work done at the Department of Biochemistry, Oxford, was aided by grants from the Rockefeller Foundation and the United States Department of Health, Education and Welfare.

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Electron Microscope Observations on the Surface Structures of *Streptomyces violaceoruber*

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SUMMARY

The surface structures of *Streptomyces violaceoruber* were studied by electron microscopy of intact organisms, carbon replicas and preparations of walls from disrupted organisms. A surface layer with a fibrillar structure was observed on the aerial hyphae and spores in all three types of preparation. The fate of this layer during spore formation and germination was studied; it is a loose covering which breaks when the spores separate and when the germ tubes emerge, and it is easily lost when the organisms are disrupted. No organized surface structures were observed on the underlying hyphal or spore walls.

INTRODUCTION

We have recently studied the fine structure of the actinomycete *Streptomyces violaceoruber* (*S. coelicolor*) in electron micrographs of thin sections (Glauert & Hopwood, 1959, 1960, 1961; Hopwood & Glauert, 1960). Sectioning reveals few details of the surface structure of a specimen; in a transverse section only a small fraction of the surface is seen, while oblique sections are often difficult to interpret. Observations on thin sections have therefore been supplemented with an investigation of intact organisms, carbon replicas, and fragments of walls in preparations of disrupted organisms. The results of this investigation are described here.

METHODS

Organism. *Streptomyces violaceoruber* (*S. coelicolor*) strain A3 (2) (Hopwood, 1960).

Mounting specimens on electron microscope grids. Spores and fragments of aerial hyphae were mounted on Formvar-coated grids by gently touching the Formvar film on the surface of a 4- to 6-day colony growing on complete agar medium (Hopwood, 1960). Preparations of germinating spores and hyphae of the substrate mycelium were obtained by the method developed for mycobacteria by Brieger, Cosslett & Glauert (1954). A thin layer of cottonwool soaked in liquid medium was placed in a Petri dish and covered with a disk of filter paper. Grids which had been inoculated with spores as described above were placed on the filter paper with the Formvar film uppermost, and the dish incubated at 30°. After incubation the grids were removed, floated on distilled water to remove traces of growth medium, and

placed on clean filter paper to dry. Care was taken not to allow any medium or water to get on to the upper surfaces of the grids and so contaminate them.

Organisms that were dried in air were found to be seriously distorted (Pl. 1, fig. 3; Pl. 3, fig. 12) because they collapsed on to the support film; therefore the preparations were freeze-dried. A simple freeze-drying apparatus designed by Dr M. J. P. Canny was used. Grids carrying the specimens were placed one at a time on a copper disk of 1 in. diam. (a halfpenny) which had been previously cooled to about -195° by immersion in liquid nitrogen. After a few seconds the grids were rapidly transferred to a Quickfit test tube of internal diameter 34 mm., which was kept at a temperature of about -80° by immersion in a mixture of acetone and solid carbon dioxide in a vacuum flask. When the grids had all been transferred, the test tube was connected by a vacuum joint to a wide L-shaped tube. The horizontal limb of the tube contained a small boat filled with dry phosphorus pentoxide and its open end was closed with a Quickfit stopper of 34 mm. diameter. A side arm from the drying tube was connected to an Edwards two-stage rotary pump, Model 2S20B, which decreased the pressure in the freeze-drying apparatus to about 0.05 mm. Hg. The specimens were dried for about 16 hr.

Some preparations of intact organisms were shadowed with gold-palladium (60:40) before examination in the electron microscope.

Carbon replicas. Replicas were made by the method described by Bradley & Williams (1957) for the examination of the spores of bacilli. Freeze-dried specimens were transferred directly from the freeze-drying apparatus to the vacuum chamber for carbon evaporation. The carbon replicas were not metal-shadowed because it was found that granulation of the shadowing metal obscured the finer details of the surface structures.

Negative staining. A suspension of spores and small fragments of aerial mycelium was prepared as described by Glauert & Hopwood (1960). The organisms were disrupted by shaking the suspension in a Mickle tissue disintegrator (Mickle, 1948) with an equal volume of grade 12 ballotini for 5 min. Unbroken organisms were removed by centrifugation at 2000 rev./min. for 5 min. and the walls were collected by centrifugation at 10,000 rev./min. for 20 min. The pellet was thoroughly dispersed in a small volume of water, and the resulting suspension was mixed with an equal volume of a 2% (w/v) solution of phosphotungstic acid adjusted to pH 7.4 with N-KOH (Brenner & Horne, 1959). Minute drops of the mixture were placed with a fine pipette on electron microscope grids coated with collodion films stabilized with a layer of carbon and allowed to dry.

Electron microscopy. Observations were made with a Siemens Elmiskop I operating at 60 or 80 kV with a $50\ \mu$ objective aperture, and photographs were taken at instrumental magnifications of $\times 5000$ to 20,000 on Ilford Special Contrasty Lantern Plates.

RESULTS

Intact organisms

Information obtained from the study of intact hyphae and spores of *Streptomyces violaceoruber* in the electron microscope adds little to the knowledge gained by phase-contrast microscopy (Hopwood, 1960). The resolution of structures in the electron micrographs is low because the organisms are too thick to be penetrated

appreciably by the electron beam. The spores either appear uniformly opaque (Pl. 1, figs. 1, 2), or else, in air-dried preparations (Pl. 1, fig. 3), show light areas where the protoplasm has shrunk irregularly during drying; no significant internal structure is visible. The spines that are present on the spores of some other strains of *Streptomyces* (Flaig, Beutelspacher, Küster & Segler-Holzweissig, 1952; Baldacci & Grein, 1955) are not found in *S. violaceoruber*. Some spores have an irregular outline (Pl. 1, fig. 1), while others are smooth (Pl. 1, fig. 2); the irregular contour appears to be caused by a loose superficial layer which readily comes away, to leave the spores smooth-surfaced. Occasionally some details of the structure of this layer can be seen when it is partially separated from the spores (Pl. 1, fig. 4). In shadowed preparations it appears to consist of fibrils which are straight or slightly curved, often paired, and intersect at various angles to form a 'basket-work' pattern.

Carbon replicas

The walls of the substrate hyphae (Pl. 1, fig. 5) show no organized structure in carbon replicas; the surfaces have merely the same fine stippling, with light and dark circles, that is visible on the background. The aerial hyphae have a quite different appearance and are covered with a pattern of intersecting fibrils (Pl. 1, fig. 6), which are 5–10 m μ in diameter and 100 m μ or more long. Although often in pairs, the fibrils also occur singly, and sometimes a number of them radiate from one point; some appear beaded. Thus the aerial hyphae are covered with a fibrous layer which in thin sections (Glauert & Hopwood, 1961) can be seen to form a loose covering outside the wall of the hypha proper. The fibrous layer remains intact while the spores are forming (Pl. 2, figs. 7, 8), but as they begin to separate from one another, the outer layer of the wall of the parent hypha ruptures between adjacent spores (Glauert & Hopwood, 1961) and with it the fibrous layer, so that the underlying and apparently structureless spore wall is revealed (Pl. 2, fig. 9, SW; Pl. 3, fig. 11, SW). Each spore in a chain is surrounded by a sac consisting of the outer component of the parent hyphal wall, overlaid by the fibrous layer. Sometimes this sac is loose-fitting (Pl. 2, fig. 8) and sometimes it closely invests the mature spore (Pl. 2, fig. 10). As the spore germinates, the germ tubes, with apparently structureless walls, emerge through the sac (Pl. 3, fig. 13; Pl. 4, fig. 14).

Negative staining

Observations on fragments of cell walls in preparations of disrupted organisms examined by the negative staining technique of Brenner & Horne (1959) confirmed the results obtained from studies of carbon replicas. The surfaces of the aerial hyphae and the spores showed no defined structural pattern except in the regions where fragments of the fibrous layer were still present (Pl. 4, fig. 15). The fragility of the superficial fibrous layer is evident in these preparations; it is usually lost during the disruption of the organisms in the Mickle tissue disintegrator.

DISCUSSION

The negative staining technique of Brenner & Horne (1959) did not demonstrate fine structure within the walls of *Streptomyces violaceoruber*, although the technique is capable of very high resolution, and has revealed an ordered array of minute sub-units in the walls of certain large cocci (Drs M. J. Thornley and R. W. Horne,

personal communication; Professor R. G. E. Murray, personal communication). Spherical subunits have been seen in electron micrographs of the walls of some bacteria (Houwink, 1953, 1956; Labaw & Mosley, 1954, Salton & Williams, 1954; Van Iterson, 1954) even without the use of the negative staining technique. Thus it seems that the surfaces of the walls of *S. violaceoruber* are smooth, and that the subunits seen in electron micrographs of thin sections (Glauert & Hopwood, 1961) do not give rise to irregularities on the surface. It remains to be seen whether they can be made visible in unsectioned preparations by controlled degradation of the wall.

In electron micrographs of intact organisms (Pl. 1, figs. 1, 2), the spores appear smooth or slightly irregular, and occasionally it can be seen that the irregularity is due to a detachable fibrous layer which covers the smooth spore wall. Vernon (1955) described the fine structure of a similar layer in a metal-shadowed preparation of an aerial hypha of an unnamed streptomycete, and stated that it consisted of narrow pointed plates lying side by side in groups at various angles, but he did not recognize the distinction between this fragile superficial layer and the structureless wall of the hypha underneath. The fibrous layer can be seen in electron micrographs of thin sections (Glauert & Hopwood, 1961) as a coat covering the double-layered wall of the aerial hyphae. During sporulation only the inner component of the wall gives rise to the wall of the spores; the outer component remains as a sac partially surrounding the mature spores, and is still covered by the fibrous layer.

The comparatively simple technique of carbon replicas (Bradley & Williams, 1957), particularly when combined with freeze-drying, greatly increases the amount of structure observable on the surfaces of bacteria. In electron micrographs of carbon replicas of *Streptomyces violaceoruber*, details of the structure of the fibrous coat and its behaviour during sporulation are clearly seen. The nature of the surfaces of streptomycete spores as seen in electron micrographs (that is whether they are smooth or bear projections of various forms) has recently been used as a character for the classification of the group (see Tresner, Davies & Backus, 1961, for references). Perhaps carbon replicas would enable differences to be detected amongst the many strains which have smooth spores, and might also reveal more clearly the structure of the spiny or hairy processes present in certain strains. It is probable that many of these processes are part of a superficial layer (Welch & Lechevalier, 1960) comparable to the fibrous coat of *S. violaceoruber*, rather than projections of the spore wall proper. The significance of the superficial layer, whether it bears processes or not, is unknown; possibly it is responsible for the difficulty with which streptomycete spores are wetted by water, a property that may enable them to be dispersed on air-water interfaces in the upper layers of the soil, rather than sink to lower regions. No structures comparable to the fibrils of the coat of *S. violaceoruber* have been reported on the surfaces of other bacteria; the 'paired fibrous structure' found in lysed mycobacteria by Takeya, Mori, Koike & Toda (1958) had dimensions very similar to those of the fibrils in *S. violaceoruber*, but this component was stated to be on the inside of the walls.

One of the authors (A.M.G.) is a Sir Halley Stewart Research Fellow.

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Note added in proof. In a recent paper J. Yamaguchi (*Rep. Res. Inst. Tub. & Leprosy*, **9**, 125, 1960) describes a similar fibrous layer which she considers to be on the outer surface of mycobacterial cell walls.

EXPLANATION OF PLATES

The scale marks represent 0.5 μ .

PLATE 1

Figs. 1 to 4. Electron micrographs of intact spores of *Streptomyces violaceoruber* shadowed with gold/palladium.

Fig. 1. Freeze-dried spores with rough outlines. $\times 36,000$.

Fig. 2. Freeze-dried spores with smooth outlines. $\times 36,000$.

Fig. 3. Air-dried spores which are distorted. $\times 30,000$.

Fig. 4. The fibrous layer separated from the surface of a spore. $\times 36,000$.

Figs. 5 and 6. Electron micrographs of carbon replicas of freeze-dried hyphae of *Streptomyces violaceoruber*.

Fig. 5. Part of a hypha of the substrate mycelium with a structureless surface. $\times 66,000$.

Fig. 6. Part of a hypha of the aerial mycelium with an outer fibrous layer. Some of the fibrils appear beaded. $\times 66,000$.

PLATE 2

Figs. 7 to 10. Electron micrographs of carbon replicas of freeze-dried spores of *Streptomyces violaceoruber*.

Fig. 7. The spores in a chain are covered with the fibrous layer. $\times 66,000$.

Fig. 8. The spores in a chain are surrounded by a loose sac derived from the parent hyphal wall. The spores and sac are covered with the fibrous layer. $\times 40,000$.

Fig. 9. The spores in a chain are beginning to separate from one another and the fibrous layer has broken between the spores, revealing the structureless spore wall (SW) underneath. $\times 30,000$.

Fig. 10. An isolated mature spore is closely surrounded by the fibrous layer. $\times 47,000$.

PLATE 3

Figs. 11 to 13. Electron micrographs of carbon replicas of *Streptomyces violaceoruber*.

Fig. 11. Two freeze-dried spores are separating from one another and the fibrous layer has broken between them revealing the structureless spore wall (SW) underneath. $\times 55,000$.

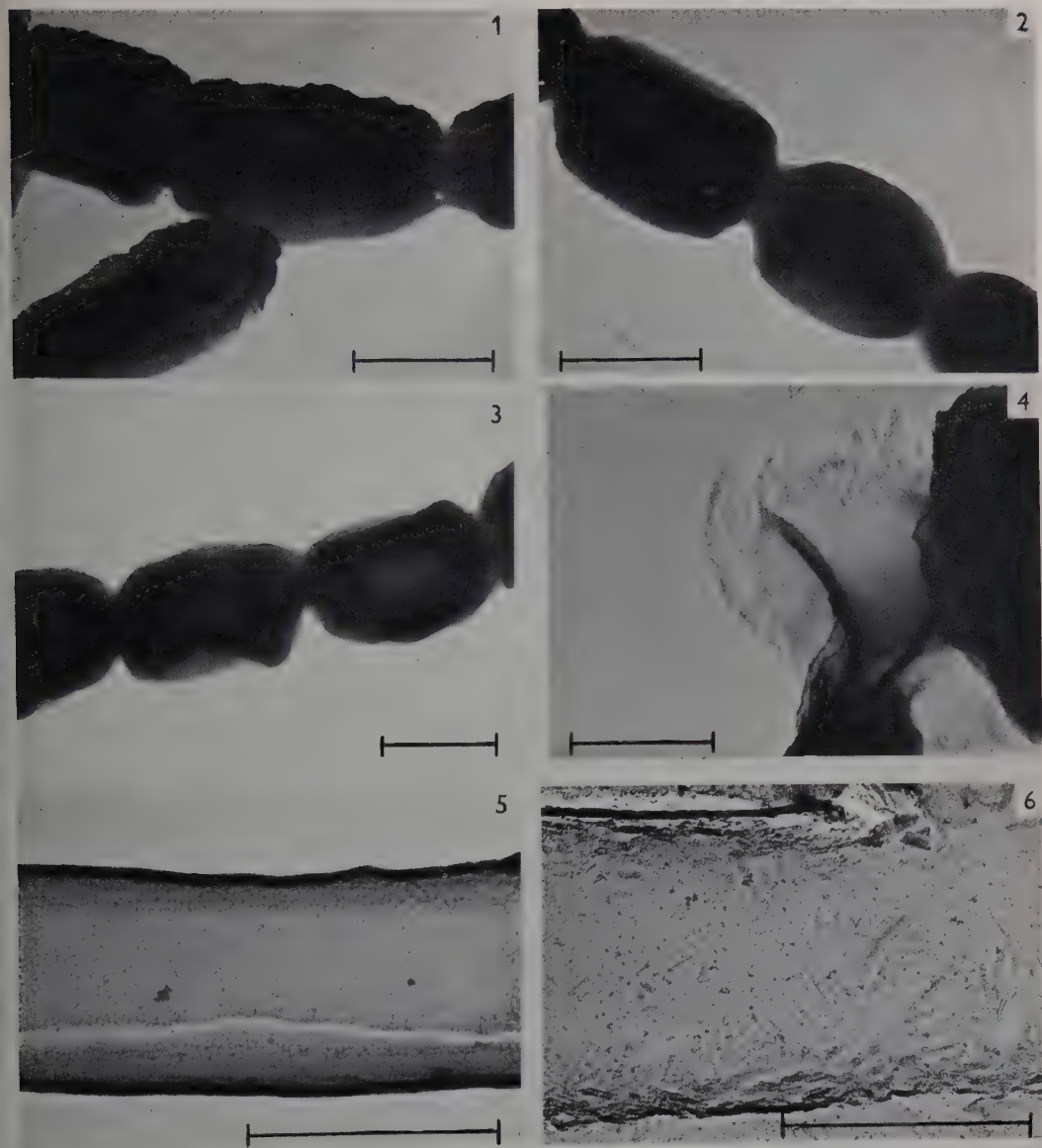
Fig. 12. An air-dried spore which is distorted. $\times 35,000$.

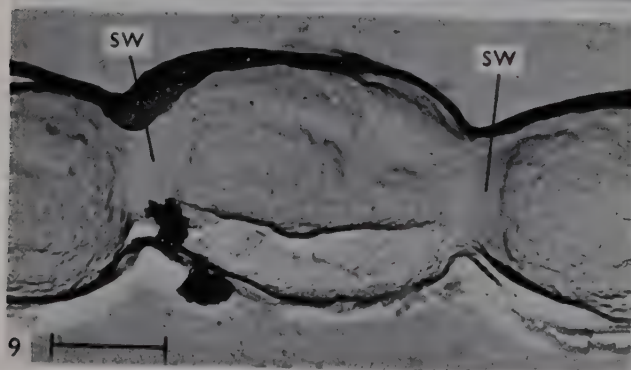
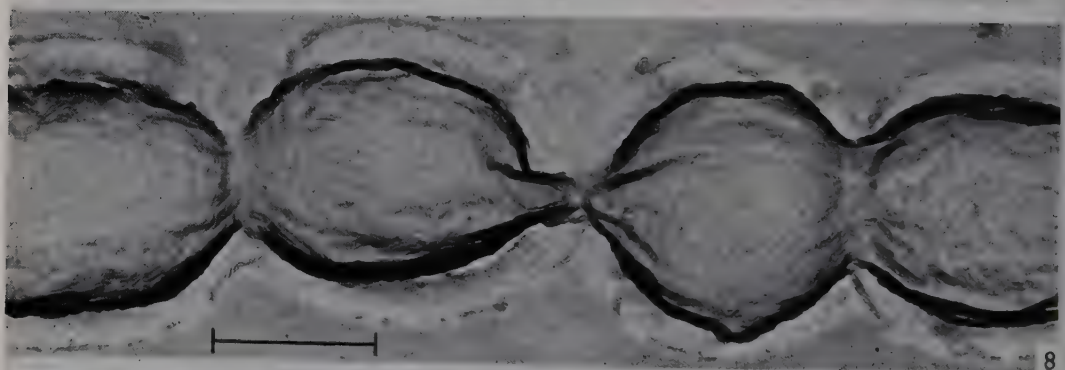
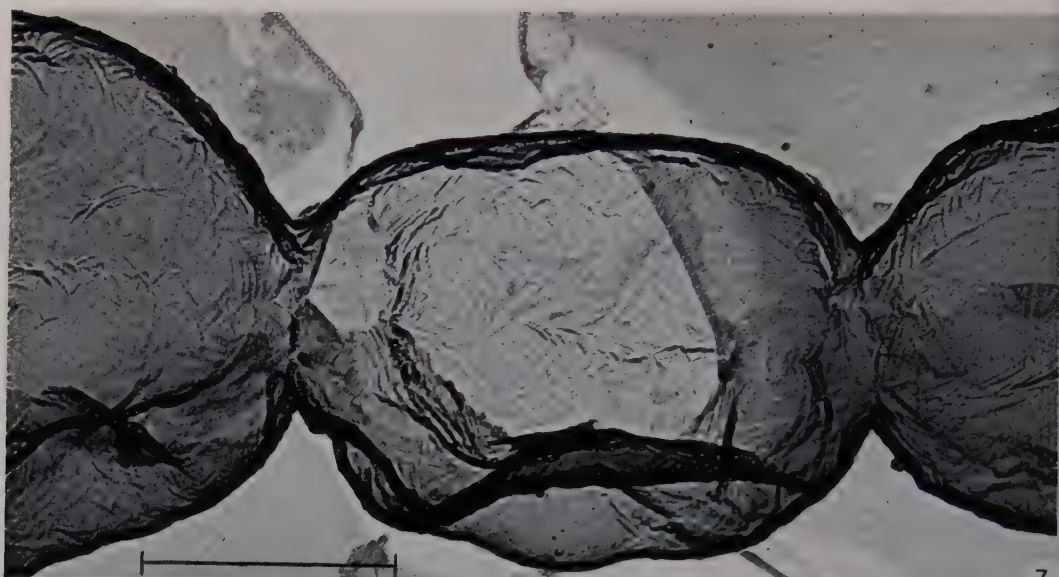
Fig. 13. A spore has just started to germinate and the short germ tube has emerged through the fibrous layer; freeze-dried. $\times 41,000$.

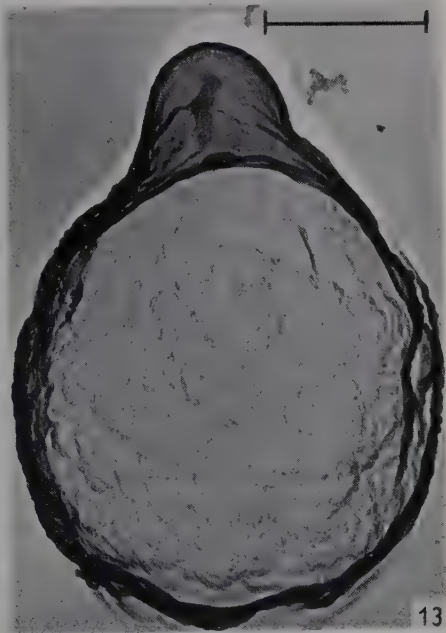
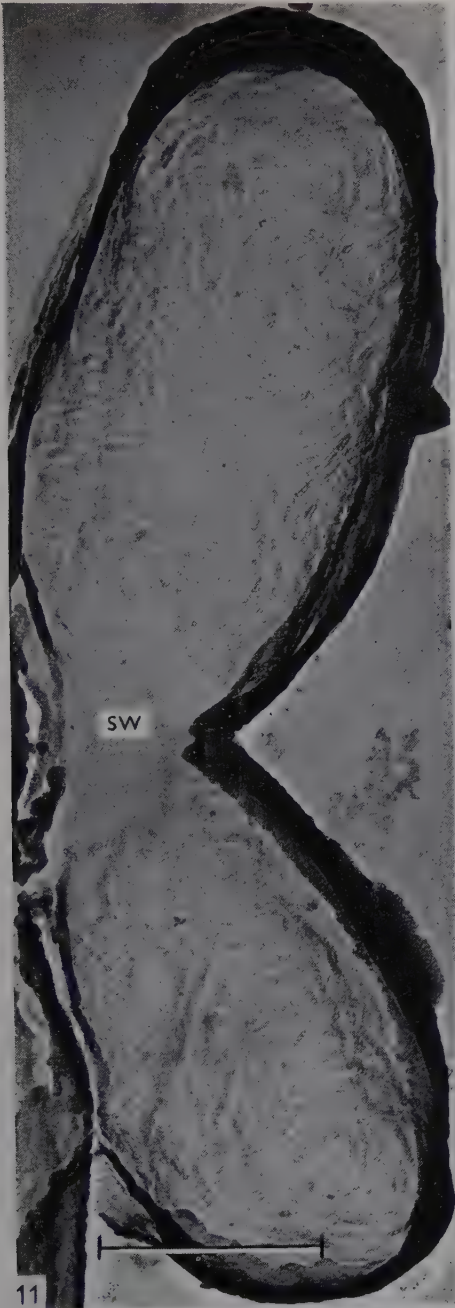
PLATE 4

Fig. 14. Electron micrograph of a carbon replica of *Streptomyces violaceoruber*. A group of spores has germinated and each has produced one or two germ tubes. The surfaces of the walls of the germ tubes show no structure, although the fibrous layer is still present on the spores. $\times 18,000$.

Fig. 15. Electron micrograph of a fragment of the wall of an aerial hypha of *Streptomyces violaceoruber* stained with potassium phosphotungstate. The hyphal wall has no visible structure. Part of the fibrous layer still covers one branch of the hypha. $\times 30,000$.

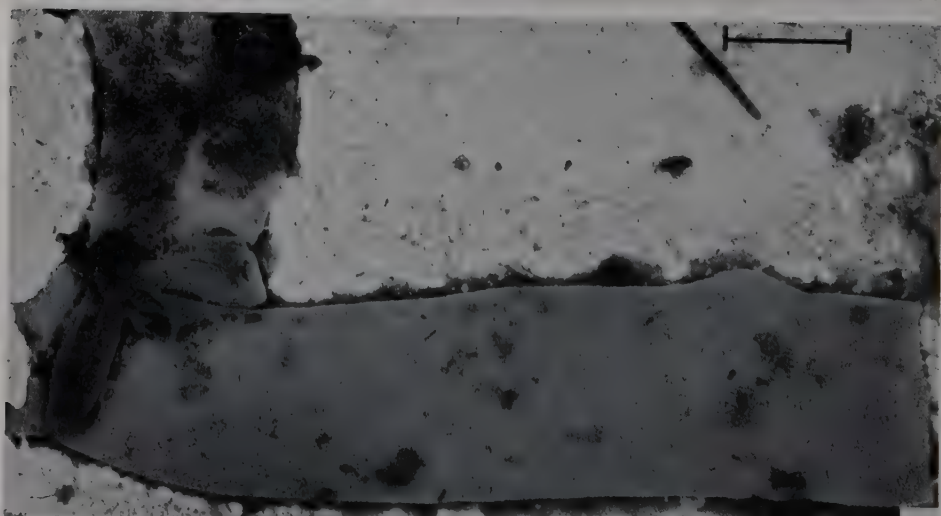








14



15

A Study of Two Marine Agar-Decomposing, Facultatively Anaerobic Myxobacteria

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(Received 19 April 1961)

SUMMARY

Two facultatively anaerobic agar-decomposing myxobacteria were isolated from marine mud and described as *Cytophaga fermentans* Bachmann var. *agarovorans* var. nov. and *C. salmonicolor* var. *agarovorans* var. nov. A third organism which does not attack agar was isolated from the same source; it has been included in the present study for comparative purposes. The specific name *Cytophaga salmonicolor* has been given to this organism. All organisms are characterized by exhibiting flexing and gliding motility and by absence of microcysts and fruiting bodies. The organisms can be grown in a mineral glucose medium, although growth is accelerated and more abundant when a vitamin mixture is included in the medium. CO₂ is an absolute requirement for growth. Growth of *C. fermentans* var. *agarovorans* and *C. salmonicolor* var. *agarovorans* on aerobic plate cultures is markedly inhibited by 0.1 % glucose or galactose. Acetic, propionic and succinic acids are major end products of sugar fermentation of all three types. *C. salmonicolor* and *C. salmonicolor* var. *agarovorans* form in addition CO₂ and H₂, and small amounts of lactic and formic acids and ethanol.

INTRODUCTION

Agar, obtained by hot water extraction of marine Rhodophyta (Mori, 1953), contains two polysaccharides, agarose and agarpectin (Araki, 1956). The latter, a minor constituent, is a rather complex substance, comprising sulphuric and uronic acid components. The major constituent, agarose, seems to consist of alternately repeated units of 1:3-linked β -D-galactopyranose and 1:4-linked 3:6-anhydro- α -L-galactopyranose; it can be enzymically hydrolysed to neo-agarobiose, which is composed of D-galactose and 3:6-anhydro-L-galactose (Araki & Arai, 1956; Yappe, 1957).

Although several kinds of agar-decomposing bacteria have been isolated from soil, such organisms represent only a very small fraction of the soil microflora; hence they are rarely encountered by the soil microbiologist. On the other hand, agar-digesting bacteria are common in marine environments. Undoubtedly this is due to the wide distribution and abundance of agar-containing seaweeds. Humm (1946), studying the marine agar-decomposing bacteria of the South Atlantic coast of the U.S.A., reported that the intertidal zone of the beaches contains 2-20 million agar-digesting bacteria per gram; as determined by plate counts, they represent 2-4 % of the total number of aerobic bacteria in this zone.

Among the 20 agar-decomposing bacteria Humm (1946) describes is *Cytophaga*

sensitiva, isolated directly from decaying marine algae. Stanier (1941) had previously discovered two other agar-decomposing cytophagas, *C. krzemieniewskae* and *C. diffluens*; these had been found on seawater count plates. These three cytophagas are strict aerobes. Bachmann (1955) was the first to describe a facultatively anaerobic cytophaga, *C. fermentans*. It produces craters and gelase fields on seawater agar plates with 1 % (w/v) yeast extract, but does not grow in media containing agar as sole energy source, even if they are adequate for growth when supplemented with a fermentable sugar. And, because galactose is also unsuitable as a carbon source for this bacterium, Bachmann (1955) concluded that it cannot be considered as a genuine agar-decomposer in the sense of an organism that can grow at the expense of agar. Anderson & Ordal (1961*a*) described another facultatively anaerobic cytophaga, *C. succinicans*, which was isolated from fresh water.

In 1947 and 1948, Drs S. R. Elsdén and H. Larsen, respectively, isolated facultatively anaerobic cytophagas, which rapidly decompose agar and produce a pink pigment, from elective cultures of green sulphur bacteria at the Hopkins Marine Station of Stanford University, Pacific Grove, California, U.S.A. A similar organism was isolated in 1958 by Dr K. Eimhjellen in the same laboratory (Dr C. B. van Niel, personal communication). The facultatively anaerobic cytophagas described in the present paper were isolated in the same institution by Dr June Lascelles, who noticed their incidental occurrence in crude cultures of purple sulphur bacteria. She also showed, as had previously been done by Elsdén, that such organisms can be more specifically enriched by using anaerobic cultures with media in which agar represents the sole carbon and energy source.

RESULTS

Isolation and maintenance

The medium used for the elective cultures contained, per 100 ml. H₂O:NaCl, 3 g.; KH₂PO₄, 0.1 g.; NH₄Cl, 0.1 g.; MgCl₂.6H₂O, 0.05 g.; CaCl₂, 0.004 g.; NaHCO₃, 0.5 g.; Na₂S.9H₂O, 0.01 g.; Fe-citrate, M/250, 0.5 ml.; trace element mixture, 0.2 ml.; powdered agar, 0.5 g.; Difco yeast extract, 0.03 g.; adjusted to pH 7.0. The trace element mixture used was that of Kohlmeier & Gest (1951).

Bottles, completely filled with this medium, were inoculated with marine mud from areas with decayed algae, stoppered, and incubated in the dark at 30°. After 3–5 days, anaerobic plate or shake cultures were prepared, using the above medium with 2 % (w/v) agar; the shake cultures were covered with paraffin to maintain anaerobiosis.

Colonies which on microscopic examination exhibited the flexing movements characteristic of myxobacteria were further purified by the shake culture method.

The isolates were maintained as paraffin-covered stab cultures in the elective culture medium with 1 % (w/v) agar, supplemented with 0.1 % (w/v) glucose for *Cytophaga salmonicolor* and as anaerobic liquid cultures in Hall tubes as modified by Barker (1936) in such a medium in which the agar was replaced by 0.1 % (w/v) glucose, galactose or starch. The cultures were incubated at 30° for 2–3 days, and thereafter stored at 4°; transfers were made monthly.

For aerobic plate and semi-anaerobic stab cultures a modified agar medium was used; it contained only 0.05 % NaHCO₃, no Na₂S, and 0.1 % each of yeast extract, corn steep liquor, and dehydrated nutrient broth.

Growth requirements

All three strains grew equally well anaerobically in the liquid medium when the yeast extract was replaced by a mixture of pyridoxin, riboflavin, thiamine, nicotinamide, pantothenic acid, folic acid and *p*-aminobenzoic acid, each at 0.4 mg./l.; biotin, 0.04 mg./l.; and cyanocobalamine, 0.002 mg./l. They may also grow in a vitamin-free glucose medium; but here growth is far less copious and sometimes erratic, particularly in the case of semi-anaerobic cultures of *Cytophaga fermentans* var. *agarovorans*. In contrast, aerobic cultures did not develop on chemically defined media. *C. fermentans* var. *agarovorans* and *C. salmonicolor* var. *agarovorans*, which both decomposed agar extensively under anaerobic conditions, grew at best sparsely

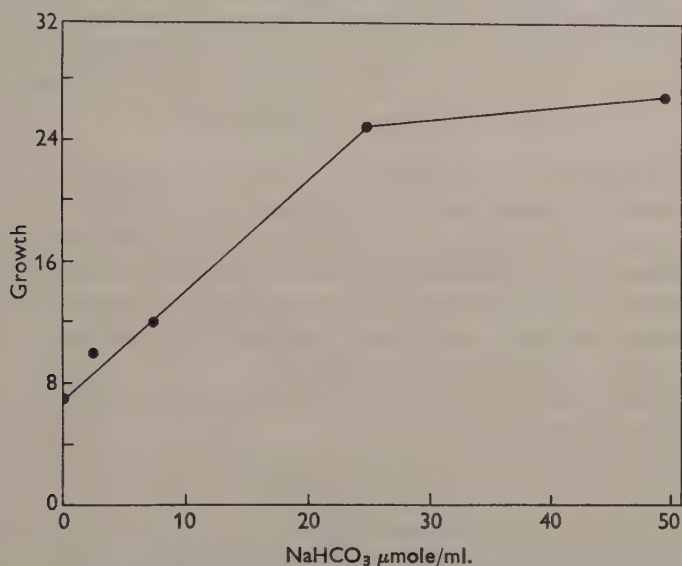


Fig. 1. Growth of *Cytophaga fermentans* var. *agarovorans* in mineral medium with 0.1 % (w/v) mannitol+0.03 % (w/v) yeast extract at different NaHCO₃ concentrations. Turbidity measured after cultivation for 2 days by Eel colorimeter. Data provided by Dr June Lascelles.

on a 1 % (w/v) agar medium with 0.1 % (w/v) yeast extract. However, when this medium was supplemented with corn steep liquor and nutrient broth, these organisms grew well and formed deep craters in the agar solidified medium, showing that they digested agar aerobically also.

Bachmann (1955) observed a similar phenomenon with *Cytophaga fermentans*. Anaerobically this organism required thiamine as the only growth factor; but it did not grow aerobically unless 1 % (w/v) yeast extract was added. Then growth was accompanied by a softening of the agar and the appearance of shallow craters and gelase fields. In the absence of air this species did not grow with agar as sole C source.

Additional information about growth of our organisms on agar will be found below. A wide variety of carbohydrates is fermented by these organisms; they are listed under the respective species descriptions. The pH optimum for growth is about

7-7.5; fermentation ceased when the pH value decreased to 5.5, after which the cultures became non-viable within a week. All strains grew rapidly at temperatures between 28° and 37°. Growth under anaerobic conditions was very poor when NaHCO_3 was omitted from the medium; Fig. 1 shows the effect of different NaHCO_3 concentrations. It is evident that an optimal response was reached at a concentration of about 0.3 %. For these experiments a heavily buffered medium was used; hence it seems unlikely that the bicarbonate exerted its influence by regulating the pH value of the medium. Because our cytophagas carry out a propionic acid fermentation, which in other cases has been shown to involve carboxylation reactions, it seems far more probable that a fairly high CO_2 tension is required for metabolic activity. If CO_2 were also indispensable for aerobic growth, the need to supplement agar media with yeast extract, corn steep liquor and nutrient broth may be explained by postulating that on such enriched media an adequate CO_2 tension is produced locally. This opinion is supported by the fact that aerobic growth of the cytophagas may occur at the expense of the complex nitrogenous compounds alone; but the addition of a fermentable carbohydrate speeds up and enhances the development.

The fermentation of glucose by *Cytophaga succinicans* also appears to be CO_2 -dependent (Anderson & Ordal, 1961*b*). From their experiments with this organism these authors concluded that ' CO_2 functions in the fermentation by providing, through condensation with phosphoenolpyruvate, compounds which can serve as acceptors for the available hydrogen generated in glucose degradation.'

Nitrates, ammonium salts, yeast extract, nutrient broth, and Casamino acids also can serve as nitrogen source for our cytophagas.

All strains grew well in media with 1-3 % (w/v) NaCl; very little growth occurred at lower NaCl concentrations.

Morphology

Our cytophagas could not be differentiated on the basis of their morphology. The organisms of all strains were slender with rounded ends (Pl. 1, fig. 5); they were extremely flexible and weakly refractile, and exhibited gliding movements, often alternately forwards and backwards. These movements were generally rather slow and regular, although sometimes sudden 'jumps' were observed. In wet mounts the cells were often seen to move against currents in the liquid. Organisms embedded in agar were generally much coiled. These organisms cannot be considered as involution forms since they straightened out and often appeared to be actively motile when forced into liquid channels surrounding the agar in wet mounts.

The organisms varied from 2 to 30 μ in length; occasionally very long elements, up to 50 μ long were seen. These long organisms, which also occurred in young cultures often showed flexing and gliding movements. The average length of organisms grown aerobically on agar plates was usually greater than that of organisms from stab or anaerobic liquid cultures. Short cells (about 3 μ long) did not show flexibility. They were never found to predominate, however, as they do in cultures of *Cytophaga johnsonae* (Stanier, 1947). Branched cells were never observed in our cultures. Fruiting bodies were not observed, nor were microcysts. Thus our strains are typical representatives of the genus *Cytophaga* as defined by Stanier (1942).

In cultures which had reached the stationary growth phase, spherical organisms

(Pl. 1, fig. 6) were always found. They varied in diameter from 1 to 3.5 μ , and small round bodies or rod-like structures could usually be seen within these organisms. Rod-shaped organisms with a spherical extrusion also frequently occurred in these cultures.

The fact that the spherical cells varied considerably in diameter, as well as the observation that they were formed when cultures were about to lose their viability, makes it seem unlikely that they were microcysts as formed in the genus *Sporocytophaga*. Bachmann (1955) reached similar conclusions about the round bodies she observed in aged cultures of *Cytophaga fermentans*. Coccoid involution forms were also found in cultures of *C. hutchinsonii* and *C. krzemieniewskae* (Stanier, 1942) and *C. succinicans* (Anderson & Ordal, 1961a). Spherical bodies reminiscent of those encountered in the *Cytophaga* cultures have also been found in *Treponema zuelzeriae* (Veldkamp, 1960). Attempts to show the viability of these spherules failed. The transformation into spherical bodies in dying organisms might be a common characteristic of organisms which lack a rigid cell wall.

Growth on agar media

Anaerobic growth. Plate 1, fig. 1, shows large spherical colonies of *Cytophaga fermentans* var. *agarovorans* developing in an agar shake culture (1 % agar, 0.1 % yeast extract.) The organisms in these cream-coloured colonies were embedded in the softened agar. In the clear zone which surrounded the colonies the agar was softened by extracellular enzymic activity. *C. salmonicolor* var. *agarovorans* produced pink colonies of similar shape; the area occupied by these colonies consisted of completely liquefied agar. The shape of the colonies was not influenced by the inclusion of 0.1 % (w/v) galactose in the medium; but the colonies were then slightly more dense.

Cytophaga salmonicolor grew very poorly in the above medium unless cornsteep liquor and nutrient broth were added (0.1 %, w/v, each); spherical, pinkish colonies were then formed which never exceeded 1–2 mm. in diameter (Pl. 1, fig. 2). Softening of the agar was not observed. The addition of glucose or galactose to the medium yielded denser spherical or disk-shaped salmon-coloured colonies of the same size.

Stab cultures of *Cytophaga fermentans* var. *agarovorans* and *C. salmonicolor* var. *agarovorans* in a medium with 1 % agar and 0.1 % yeast extract are illustrated in Pl. 1, figs. 3, 4. Growth gradually spread outward from the inoculated region, as it did in the case of a colony developing in an agar shake culture (Pl. 1, fig. 1). This is attributable to the softening (*C. fermentans* var. *agarovorans*) or liquefaction (*C. salmonicolor* var. *agarovorans*) of the agar, which permits the cells to migrate. Thus the periphery of a growing colony or stab culture often appears as a diffuse area of low optical density, representing the zone of migration. In media with 2 % (w/v) agar the tendency to spread was considerably decreased. In stab cultures of *C. fermentans* (ATCC no. 12470) and *C. salmonicolor* the migration of organisms was limited to a distance of 1–2 mm. from the line of inoculation when 1–1.5 % (w/v) agar was used; decomposition of agar was not observed.

Aerobic growth

As stated above, all strains grew copiously under aerobic conditions on a mineral medium containing 1 % (w/v) agar and 0.1 % (w/v) each of yeast extract, corn steep

liquor or nutrient broth. *Cytophaga fermentans* var. *agarovorans* formed greyish to pale yellow colonies which attained a diameter of about 3 cm. on sparsely seeded plates. The colonies formed deep depressions in the agar and were surrounded by a zone in which the agar became translucent, softened, and generally slightly depressed (Pl. 2, fig. 8). On flooding the plate with a I + KI solution (a test devised by Gran, 1902) this zone remained unstained (Pl. 2, fig. 9). Colonies of *C. fermentans* var. *agarovorans* which developed on the above medium were largely subsurface, and generally had a sharp even edge. Occasionally swarming growth across the agar surface was observed, although this was generally limited to small sections of a colony. On media with 2 % (w/v) instead of 1 % agar, where the penetration of the organisms into the agar was impeded, growth occurred as flat spreading colonies, crater formation was less rapid and the depressions remained very shallow. The influence of agar concentration on the extent of penetration was reminiscent of Stanier's (1947) observations with *C. johnsonae*.

Cytophaga fermentans var. *agarovorans* tended to produce colonial variants. Two such variants were encountered: one formed relatively small bright yellow colonies of the sunken type; the other formed small raised pale yellow colonies, which hardly affected the structure of the agar. Swarming motility across an agar surface was never observed with these variant strains. The organisms in the aberrant colonies were morphologically similar to those of the parent strain. Similar variations in colony type were observed by Bachmann (1955) with *C. fermentans*. When grown on a medium containing 1 % (w/v) agar, *C. salmonicolor* var. *agarovorans* produced salmon-coloured colonies surrounded by wide gelase fields; the diameter of the colonies generally did not exceed 1 cm. The depressions formed on the agar surface were usually not so deep as those formed by *C. fermentans* var. *agarovorans*; although the organisms migrated into the agar, this tendency was less marked than with *C. fermentans* var. *agarovorans*. The agar did not become completely liquefied as in anaerobic cultures. *C. salmonicolor* var. *agarovorans* sometimes exhibited spreading growth on aerobic agar plates. Some colonies were apt to present a swarming edge, others a smooth sharply defined circumference. Either colony type yielded both forms on replating. A similar case of colony dimorphism was observed by Stanier (1942) with *Sporocytophaga myxococcoides*. Unlike *C. fermentans* var. *agarovorans*, *C. salmonicolor* var. *agarovorans* generally showed a decreased degree of swarming on a medium with 2 % (w/v) agar; crater formation was then slightly retarded.

Cytophaga salmonicolor, like *C. salmonicolor* var. *agarovorans*, formed salmon-coloured colonies with a maximum diameter of 1 cm. They were either flat and spreading (Pl. 1, fig. 7) or convex with a smooth edge. When grown on a 1 % (w/v) agar-medium, the organisms penetrated to a limited extent into the agar, and the colonies occasionally sank slightly into the agar. Gran's test was weakly positive (max. diam. of zone around colonies 0.5 mm.), but the agar around the colonies was never depressed or softened, as was the case with the two other types. Colonies never appeared sunken on a medium with 2 % (w/v) agar. Fermentation tests carried out with washed agar as a carbon source showed that *C. salmonicolor* did not ferment agar.

The above-mentioned observations lead to the conclusion that *Cytophaga salmonicolor* was unable to grow with agar as sole C source. Colonies of aerobically grown *C. salmonicolor* were catalase positive. *C. salmonicolor* var. *agarovorans* and

C. fermentans var. *agarovorans* sometimes showed a negative catalase reaction when grown on a medium containing 1% (w/v) agar, even when CaCO_3 was included. When 2% (w/v) agar was used the tendency to penetrate into the agar was less pronounced; in this case the agar-decomposing cytophagas generally gave a positive catalase reaction.

Inhibitory effect of glucose and galactose

Under semi-anaerobic conditions (stab cultures in agar media without Na_2S and without paraffin seal), or on aerobic plates, glucose and galactose markedly inhibited growth of *Cytophaga fermentans* var. *agarovorans* and *C. salmonicolor* var. *agarovorans*. Only a minute fraction of the inoculated viable organisms produced colonies in or on the sugar-containing substrate, and these colonies appeared where the inoculum was densest. These colonies were similar to those which grow on sugar-free media, and they decomposed agar at nearly the same rate. The inhibition by glucose and galactose could not be attributed to the formation of toxic products during autoclaving; when sterilized by Seitz filtration and added to the agar after this had been cooled to 40°, solutions of these sugars exerted the same effect. The growth of *C. salmonicolor* on agar media was hardly affected by adding glucose; but the addition of 0.1% (w/v) galactose strongly inhibited the development of the organism under aerobic conditions. A similar behaviour was reported for the agar-decomposing *C. sensitiva* (Humm, 1946), which did not grow on agar media with added 0.2% (w/v) glucose or starch; and for the cellulose-decomposing *Sporocytophaga myxococcoides*, which also was prevented from growing by 0.2% (w/v) glucose (Kaars Sijpesteijn & Fåhræus, 1949). The mechanism of this striking inhibition is still obscure.

The nature of the fermentation

Methods. Fermentations were carried out in Hall flasks or in an apparatus previously described (Veldkamp, 1960); the latter apparatus was modified to maintain a constant pH value during the fermentation by means of an automatic titrator type TTT1 (Radiometer, Copenhagen, Denmark). For this purpose the all-glass fermentation vessel was provided with ground glass sockets for the glass- and calomel electrodes and alkali inlet. During the fermentation sterile NaOH was added from a burette operated by a magnetic valve.

The electrodes were sterilized chemically using a commercial iodophor solution (JO 127; Amsterdamse Kininefabriek, Amsterdam, Netherlands) which contains 1.5% active iodine. The electrodes were immersed for 20 min. in a 1/500 dilution of the iodophor solution and then washed with sterile water.

The experiments were made at 30° in an oxygen-free nitrogen atmosphere, with a mineral medium which in addition to the usual salts contained (% w/v) 0.1, NaHCO_3 ; 0.05, $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$; adjusted to pH 7.

For the fermentations with agar as carbon source, 0.3% (w/v) Difco agar was used (this had been carefully washed with distilled water for 7 days); 0.1% (w/v) each of yeast extract, corn steep liquor and nutrient broth were included. Control experiments showed that only a trace of acid was formed when the agar was omitted from the medium. For hexose fermentations 0.1% (w/v) yeast extract and either 0.1–0.2% (fermentations in Hall flasks) or 1% sugar (fermentations at constant pH) were added to the basal medium. The sugars were sterilized separately as concen-

trated aqueous solutions. The analyses of fermentation products were performed as previously described (Veldkamp, 1960).

The dry weight of the slimy cell material was determined as follows. At the end of the fermentation 3 volumes of ethanol were added to a sample of the culture liquid; the resulting stringy precipitate was centrifuged down, washed twice with 95 % (w/v) ethanol in water, then dried *in vacuo* at 45° and weighed. This material was considered to have the empirical formula (CH₂O), an approximation supported by studies of bacterial assimilation (see Clifton, 1951).

Results. The results of the fermentations with galactose as carbon and energy source are shown in Table 1 which shows that acetic, propionic and succinic acids were the major products of galactose fermentation by all the organisms tested. *Cytophaga salmonicolor* and *C. salmonicolor* var. *agarovorans* formed a considerable amount of formic acid. Fermentations with washed Difco agar as substrate showed that *C. salmonicolor* did not form acid, whereas vigorous acid-production was observed with cultures of *C. salmonicolor* var. *agarovorans* and *C. fermentans* var. *agarovorans*, with a concomitant decrease in pH value from 7.0 to 5.4. The same acids were found in agar and galactose fermentations.

Table 1. *Acids produced during fermentation of galactose in Hall flasks by various cytophagas*

Product	<i>C. salmonicolor</i>		<i>C. fermentans</i>
	<i>C. salmonicolor</i>	var. <i>agarovorans</i>	var. <i>agarovorans</i>
	mm product/mm galactose fermented		
Formic acid	0.50	0.33	0
Acetic acid	0.42	0.26	0.27
Propionic acid	0.26	0.23	0.48
Succinic acid	0.32	0.38	0.13

A more detailed analysis of the fermentation products formed by *Cytophaga salmonicolor* var. *agarovorans* from glucose is presented in Table 2. This fermentation was run at pH 7.0; under these conditions 1 % glucose was completely consumed. From Table 2 it can be seen that acetic, propionic and succinic acids were the major fermentation products. In addition, considerable amounts of CO₂ and H₂ were formed; these were also formed by *C. salmonicolor*, but not by *C. fermentans* var. *agarovorans*. Small amounts of formic and lactic acids and of ethanol were found as end products of the glucose fermentation by *C. salmonicolor* var. *agarovorans*. As much as 21 % of the glucose consumed was converted to cell material (bacterial cells and slime). According to Bachmann (1955), 15–25 % of glucose consumed during fermentation of *C. fermentans* was converted to alcohol-precipitable cell material. The fermentation products formed by this organism are the same as those produced by *C. fermentans* var. *agarovorans*. It appears that all facultatively anaerobic cytophagas so far studied exhibit a propionic acid type of fermentation. *C. succinicans*, which Anderson & Ordal (1961*a, b*) showed to produce formic, acetic, and succinic acids but not propionic acid, may be considered as a variant lacking the ability to decarboxylate succinate. A similar connexion is encountered in the lactate and sugar fermentations by *Propionibacterium*, *Veillonella* and *Ruminococcus* species, respectively.

Table 2. *Fermentation of glucose at pH 7.0 by Cytophaga salmonicolor var. agarovorans*

Product	mm product/mm glucose fermented	mg. atom carbon
CO ₂	0.32	0.32
H ₂	0.44	—
Ethanol	0.16	0.32
Formic acid	0.05	0.05
Acetic acid	0.38	0.76
Propionic acid	0.31	0.93
Succinic acid	0.33	1.32
Lactic acid	0.09	0.27
Cell material and slime, as (CH ₂ O)	1.28	1.28
Total mg. atom C	—	5.25
Carbon recovery %	87.5	—
Redox index	0.96	—

Taxonomy

The following characters exhibited by our organisms indicate that they must be considered as members of the genus *Cytophaga* (Stanier, 1942): flexing and gliding motility, low-refractility, ability to swarm across an agar surface, absence of microcysts and fruiting bodies. All the strains examined were facultative anaerobes found in marine environments, characters which they have in common with *C. fermentans* (Bachmann, 1955). *C. salmonicolor* differs from *C. fermentans* by the formation of a red pigment and ability to produce formic acid, CO₂ and H₂ during fermentation of carbohydrates. Our *C. fermentans* var. *agarovorans* differs from *C. fermentans* by its ability to grow with agar as sole C source. A similar difference exists between *C. salmonicolor* and *C. salmonicolor* var. *agarovorans*. Since ability to decompose complex polysaccharides is an important characteristic in the classification of the myxobacteria, and since agar decomposition is associated with the appearance of cultures on or in agar, our agar-decomposing cytophagas are here described as varieties of *C. fermentans* and *C. salmonicolor*.

Description of *Cytophaga salmonicolor*

Morphology: flexible weakly refractile, slender rods with rounded ends. Organisms vary in length from 2 to 30 μ ; average about 6 μ . Width 0.3–0.5 μ . Gliding motility in young cultures. Coccoid involution forms common in old cultures. Branched organisms do not occur. Star-shaped aggregates of actively flexing organisms common in liquid cultures. Gram-negative.

Growth on agar plate: colonies on medium with 1 % agar and low nutrient concentration are salmon-coloured; may or may not show spreading growth across surface. Colonies sometimes slightly sunken into agar, but the agar around colonies never depressed or softened. On 2 % agar colonies never sunken into agar.

Agar shake culture: small, pink, spherical or disk-shaped colonies which do not attack agar. Apparently grows at expense of added nutrients to agar medium.

Agar stab culture: grows only when glucose or complex nitrogenous nutrients are added. Diameter of stab in 1 % agar does not exceed 2 mm.

Gelatin stab: very slow crateriform liquefaction.

Fermentation: the following substrates fermented: arabinose, xylose, glucose, galactose, mannose, fructose, sucrose, lactose, maltose, cellobiose, trehalose, raffinose, inulin, starch. Not fermented: rhamnose, sorbose, mannitol, sorbitol, agar. Chitin not attacked aerobically. Products of glucose fermentation: formic, acetic, propionic, succinic, lactic acids, CO_2 , H_2 , traces of ethanol.

Nitrogen sources: ammonium salts, nitrates, yeast extract, nutrient broth, Casamino acids.

Growth factors: grows in mineral glucose medium; heavier and more rapid growth occurs in the presence of a vitamin mixture; carbon dioxide an absolute requirement for growth.

Catalase produced.

Salt concentration range: 1.0–3.0 % (w/v) NaCl.

Facultatively anaerobic.

Optimum temperature: 28–37°.

Source: marine mud.

Habitat: probably decaying seaweeds.

Description of Cytophaga salmonicolor var. agarovorans

Morphology: similar to *C. salmonicolor*.

Growth on agar plate: colonies on a medium with 1 % agar and low nutrient concentration are salmon-coloured, sunken into the agar; may or may not show swarming across agar surface. Colonies surrounded by wide translucent gelase fields in which agar is depressed and softened; agar is not completely liquefied. Formation of depressions in agar slightly retarded when 2 % agar used.

Agar shake culture: in medium with 1 % agar, pink spherical colonies formed, up to 1 cm. diam.; agar in area occupied by colony completely liquefied.

Agar stab culture: in medium with 1 % agar as energy source diffuse growth with complete liquefaction of agar; diameter of stab 4–10 mm. Growth in 2 % agar considerably less diffuse.

All other characteristics are similar to those encountered with *C. salmonicolor* with the exception of fermentation test with washed agar as substrate (positive).

Source: marine mud.

Habitat: probably decaying seaweeds.

Description of Cytophaga fermentans var. agarovorans

Morphology: very similar to *C. fermentans* and *C. salmonicolor*.

Growth on agar plate: colonies on medium with 1 % agar and low nutrient concentration greyish; centre of colony may become cream to pale yellow. Colonies largely subsurface and form deep craters in agar; they are surrounded by wide gelase fields in which agar becomes depressed, translucent and softened. Swarming growth across agar surface may occur, although generally colonies have a sharp smooth edge. On 2 % agar media, colonies flat spreading nearly colourless translucent. In older parts of colonies organisms may tend to accumulate in drop-like masses which contain normal vegetative organisms. Depressions formed in 2 % agar very shallow.

Agar shake culture: in medium with 1 % agar, greyish to cream spherical colonies which attain a diameter of 1 cm.; agar is softened.

Agar stab culture: in medium with 1 % agar as energy source diffuse growth causing softening of agar; stabs attain diameter of 4–10 mm.; growth in 2 % agar considerably less diffuse.

Gelatin stab culture: slow stratiform liquefaction.

Fermentation: the following substrates fermented: arabinose, xylose, rhamnose, glucose, galactose, mannose, fructose, mannitol, sorbitol, sucrose, lactose, maltose, cellobiose, raffinose, inulin, starch, agar. Not fermented: sorbose, trehalose. Chitin not attacked aerobically. Products of glucose fermentation are: acetic, propionic and succinic acids.

Nitrogen sources: ammonium salts, nitrates, yeast extract, nutrient broth, Casamino acids.

Growth factors: not needed when grown anaerobically or semi-anaerobically with hexoses as carbon source. Addition of a vitamin mixture causes more rapid and abundant growth, especially when cultivated under semi-anaerobic conditions.

Carbon dioxide an absolute growth requirement.

Catalase produced.

Salt concentration range: 1.0–3.0 % (w/v) NaCl.

Facultatively anaerobic.

Optimum temperature: 28–37°.

Source: marine mud.

Habitat: probably decaying seaweeds.

Cultures of the described cytophagas have been deposited in the National Collection of Industrial bacteria, Torrey Research Station, Aberdeen, Great Britain.

I am greatly indebted to Dr June Lascelles who provided cultures of the cytophagas here described and to Dr W. Clark for supplying *C. fermentans* (ATCC no. 12470). The assistance of Miss A. van Mourik is gratefully acknowledged. My thanks are due to Professor C. B. van Niel and to Dr June Lascelles for reading and improving the text of this paper.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. *Cytophaga fermentans* var. *agarovorans*. Agar shake culture; spherical colonies in 1 % agar medium with 0.1 % each of yeast extract, corn steep liquor and nutrient broth; age 9 days.
- Fig. 2. *C. salmonicolor*. Agar shake culture; small spherical colonies grown in 1 % agar medium with 0.1 % each of yeast extract, corn steep liquor, and nutrient broth; age 9 days.
- Fig. 3. *C. fermentans* var. *agarovorans*. Stab culture after 5 days' cultivation at 30° in mineral medium with 1 % agar and 0.1 % yeast extract.
- Fig. 4. *C. salmonicolor* var. *agarovorans*. Stab culture after 5 days' cultivation at 30° in mineral medium with 1 % agar and 0.1 % yeast extract.
- Fig. 5. *C. salmonicolor* var. *agarovorans*. Phase-contrast photomicrograph of living cells from anaerobic stab culture. $\times 2340$.
- Fig. 6. *C. fermentans* var. *agarovorans*. Coccoid involution forms from agar shake culture. Phase contrast. $\times 1574$.
- Fig. 7. *C. salmonicolor*. Colonies showing flat, spreading growth on 1 % agar medium.

PLATE 2

- Fig. 8. *Cytophaga fermentans* var. *agarovorans*. Aerobic plate culture with mineral medium containing 1 % agar and 0.1 % each of yeast extract, corn steep liquor, and nutrient broth. Colonies deeply sunken into agar and surrounded by zone in which agar is softened and depressed. Age one week.
- Fig. 9. *C. fermentans* var. *agarovorans*. Same plate showing gelase fields after flooding with I-KI solution.

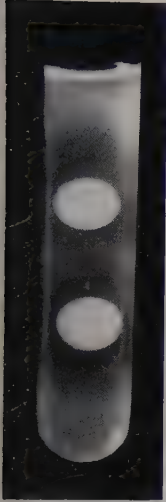


Fig. 1



Fig. 2

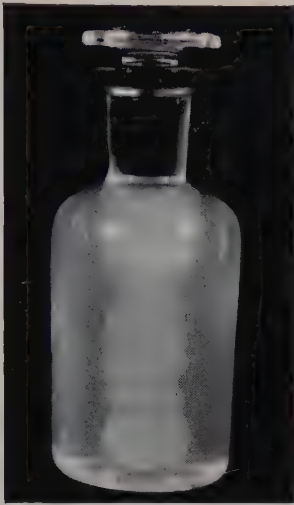


Fig. 3



Fig. 4

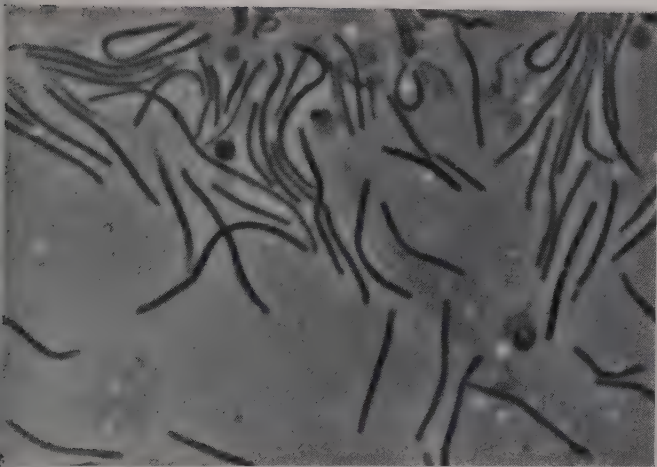


Fig. 5

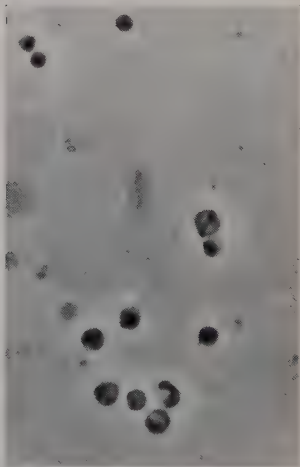


Fig. 6

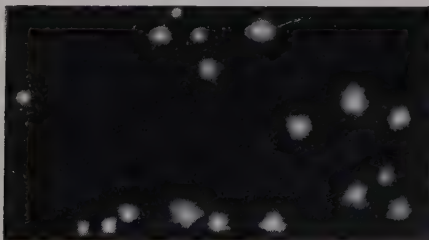


Fig. 7

H. VELDKAMP

(Facing p. 342)



Fig. 8

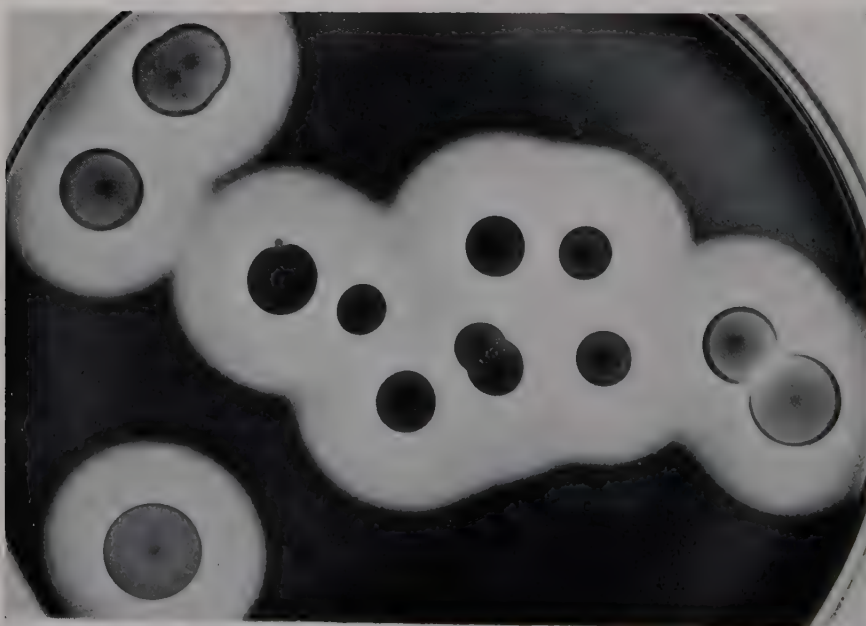


Fig. 9

Atmospheric Content of *Nigrospora* Spores in Jamaican Banana Plantations

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(Received 21 April 1961)

SUMMARY

The air in three Jamaican banana plantations was sampled from 20 July 1960 to 15 April 1961 with a Hirst spore trap. Spores of *Nigrospora* were regular components of the air-spores. They exhibited a regular and sharply defined diurnal periodicity, rapid liberation of spores starting at about 07.00 hr. and reaching a peak between 08.00 and 10.00 hr.; very few spores were trapped during the night. This is consistent with the fact that spore discharge occurs only under conditions of decreasing vapour pressure. Rainfall and under-tree irrigation usually resulted in an appreciable increase in atmospheric content of *Nigrospora* spores. These high concentrations were maintained for 2-4 days before decreasing to characteristically low dry weather values. The highest daily mean concentration recorded was 1350 spores/m.³, an estimate of *c.* 14,000/m.³ being obtained at 08.00 hr. on the same day.

INTRODUCTION

Since the introduction by Hirst (1952) of an automatic volumetric spore trap, much information about the air-spores in different parts of Britain has been collected (Hirst, 1953; Hirst, Storey, Ward & Wilcox, 1955; Gregory & Hirst, 1957; Gregory & Sreeramulu, 1958; Gregory & Stedman, 1958; Sreeramulu, 1959). In contrast, there is little information about the composition of the air-spores in or above tropical crops. Recent aerobiological studies in Jamaica (Meredith, 1961*c, d*) were chiefly concerned with the dispersal of *Deighthoniella torulosa*, the cause of banana fruit-spot (Meredith, 1961*a, b*). During this investigation it was observed that spores of *Nigrospora* were particularly common (Meredith, 1961*e*) and their dispersal is the subject of the present paper.

METHODS

Trapping methods

A Hirst spore trap with its orifice 3 m. above ground was operated continuously from 20 July 1960 to 15 April 1961; sampling was at the rate of 14.4 m.³/24 hr. Three banana plantations were selected for study, all of them being situated on the lowland plains in St Catherine where extensive banana and sugar-cane plantations are established.

Plantation A, 20 July-1 September 1960, was irrigated at fortnightly intervals by admitting water into shallow trenches (surface irrigation).

Plantation B, 4 September 1960-29 January 1961. Under-tree irrigation was carried out at intervals determined by the distribution of rainfall. In this method,

water is pumped along pipes and ejected through rotating nozzles set at intervals; pumping is usually continued for 48 hr. Since the water is sprayed several feet into the air, collapsed leaves hanging from the pseudostem are usually thoroughly wetted.

Plantation C, 15 March–15 April 1961. Conditions here were almost identical with those in B.

The slides, changed daily at 09.00 hr. E.S.T., were prepared and scanned according to the methods of Hirst (1953). Since no corrections for variation in the efficiency of trapping were made, all spore numbers quoted are underestimates (Hirst, 1953).

Daily records of temperature, R.H. and rainfall were taken in the vicinity of the trap.

Identity of Nigrospora spores

Conidia of *Nigrospora* are unlikely to be confused with those of any other genus. They are black, shiny, globose when viewed from the end and elliptical from the side. Species recorded in Jamaica include:

Nigrospora sphaerica, which is, according to Simmonds (1933), the cause of 'squirter' disease of banana fruits in Australia. It is a widespread saprophyte on banana debris and has been recorded on many other monocotyledonous hosts (Mason, 1927).

N. oryzae, a very widespread saprophyte on the banana and many other monocotyledons (Mason, 1927; Wardlaw, 1935).

N. sacchari, occurring on both banana and sugar-cane leaves (Mason 1927; Simmonds, 1933).

Mason (1927) distinguished three species of *Nigrospora* on the basis of spore size: *N. oryzae* $13-15.5 \times 10-13 \mu$, *N. sphaerica* $18-21 \times 14-15 \mu$ and *N. sacchari* $18-24 \mu$ in diameter. However, later he expressed doubt about this size criterion (Mason, 1933). In the present investigation it was found that spores measuring $13-18 \mu$ predominated on the spore traces, suggesting that the dominant species trapped were *N. oryzae* and *N. sphaerica*, *N. sacchari* being infrequent. This is consistent with some observations on the relative abundance of these species in the plantations (Meredith, unpublished).

RESULTS

Diurnal periodicity

The diurnal periodicity curve (Fig. 1) obtained according to the method of Hirst (1953) includes data from all three plantations; each mean was from a total of 224 observations. Regular periodicity was evident, rapid liberation of spores starting after 06.00 hr. and reaching a peak between 08.00 and 10.00 hr. The concentration decreased rapidly during the afternoon and evening, very few spores being trapped during the night. On a few exceptional days the peak was not reached until about 12.00 or 14.00 hr., but on no occasion was the peak reached before 08.00 hr.

Liberation coincided with conditions of rising temperature and decreasing humidity, these usually occurring from 07.00 hr. onwards until about 14.00 hr. On damp mornings following rainfall during the previous night, humidity often showed no marked decrease until about 11.00 hr.; on these occasions the peak concentration occurred after this time. Similarly, little or no liberation was evident between 08.00 and 12.00 hr. on the few occasions when it was raining at that time.

Atmospheric content of Nigrospora spores

In Fig. 2 the daily mean spore concentration of *Nigrospora* is related to locality, rainfall and under-tree irrigation over the period 20 July–15 April 1961.

Plantation A

The 4 weeks prior to commencement of sampling were predominantly dry and the daily mean concentration averaged *c.* 100/m.³. Traces of rain on 20 and 21 July were followed by an increase to 210/m.³ on 22 July. A further 0.9 in. rain on this date

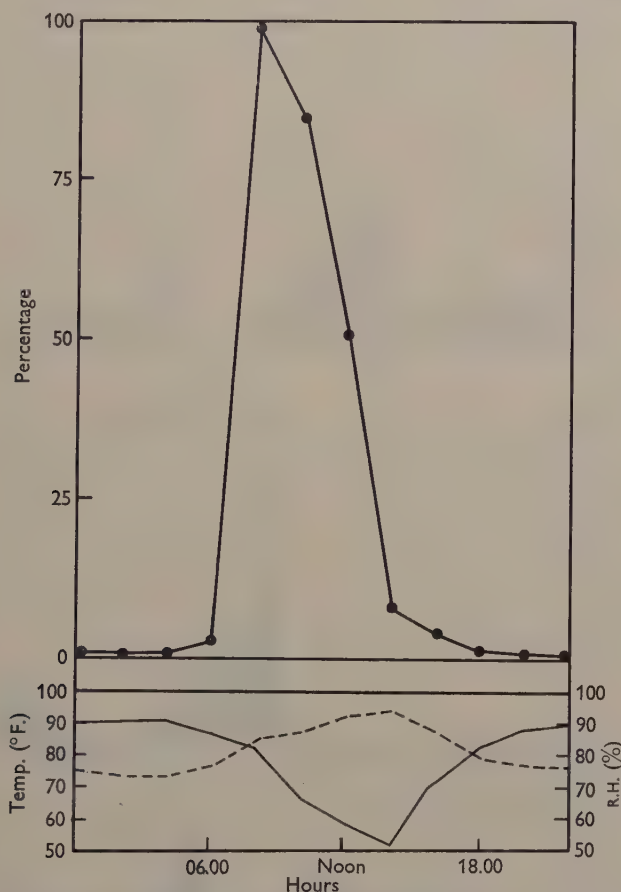


Fig. 1. Mean diurnal periodicity curve of *Nigrospora* expressed as a percentage of the peak arithmetic mean concentration. ---, temperature; —, relative humidity.

and traces on the next 2 days resulted in a concentration of 450/m.³ on 25 July. The next 7 days were dry and there was a progressive decrease in concentration to values of *c.* 100/m.³. Rainfall on various dates in August resulted in similar temporary increases in daily mean concentration. The highest 2-hourly concentration recorded in this plantation was 2400/m.³ at 08.00 hr. on 25 July.

The fifteenth of August was exceptional in that a concentration of only 6/m.³ was recorded. This was probably related to the fact that there was almost continuous

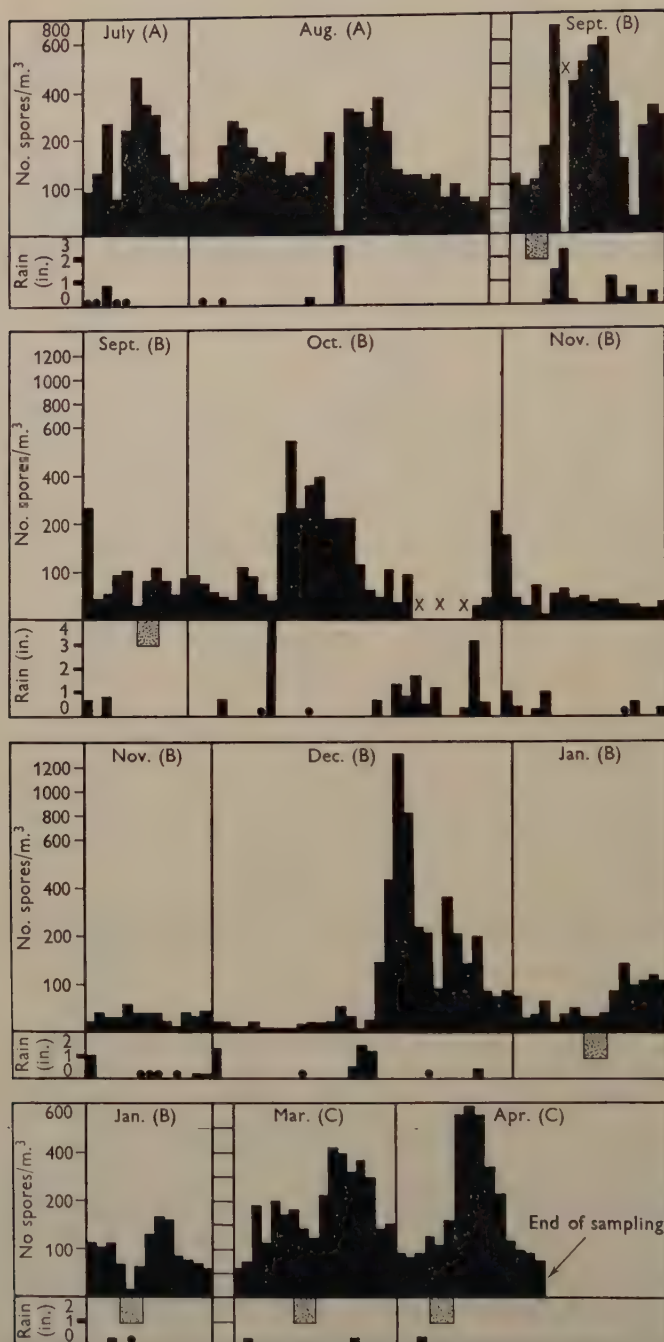


Fig. 2. Daily mean concentration of *Nigrospora* spores in three banana plantations (A, B and C) related to rain and under-tree irrigation over the period 20 July 1960 to 15 April 1961. ●, trace of rain; stippled areas indicate duration of under-tree irrigation periods; ×, trap not operating.

rainfall between 07.00 and 16.00 hr. on this date. Possibly rainfall occurring at the time of most rapid liberation removed many spores from the air almost immediately after their becoming air-borne. Alternatively, liberation might not have occurred due to unfavourable humidity conditions. A third possibility is that the source of spores was temporarily exhausted, but against this is the fact that relatively high concentrations occurred on both 14 and 16 August.

Plantation B

September. Initially, the concentration averaged *c.* 100/m.³. Under-tree irrigation was carried out from 5 to 7 September and a total of 4.13 in. rain fell between 7 and 10 September. As a result of this, the concentration increased to 636/m.³ on 8 September, an estimate of 2620/m.³ being recorded at 08.00 hr. Daily means exceeding 300/m.³ were maintained until 15 September when a value of only 30/m.³ was obtained, possibly a result of rainfall occurring between 06.00 and 10.00 hr. The next 4 days yielded counts of more than 200/m.³. Although there was more rain on 20 and 22 September and another under-tree irrigation period from 25 to 27 September, there was no appreciable increase in spore concentration.

October. The most notable feature was the greatly increased spore concentration following 4.12 in. on 9 October. The highest estimate recorded was 530/m.³ on 11 October, and it was not until 18 October that values fell below 200/m.³.

November. The concentration rarely exceeded 50/m.³, there being no apparent response to rainfall.

December. The first half of the month was predominantly dry and up to 13 December the spore concentration never exceeded 50/m.³. Rain on 15, 16 and 17 December resulted in a relatively enormous increase in concentration during the subsequent 6-day period. On 20 December a concentration of 1352/m.³ was recorded, this being the highest value recorded in any of the three plantations studied; a count of *c.* 14,000/m.³ was recorded at 08.00 hr. on this date. By 29 December the concentration had decreased to 84/m.³, there being no response to rain on 28 December.

January. This was a very dry month and concentrations exceeding 100/m.³ were recorded only after the two irrigation periods.

Plantation C

Increases in concentration were evident after rainfall and under-tree irrigation. The highest value recorded was 587/m.³ on 8 April, the peak 2-hourly estimate on this day being 5130/m.³ at 08.00 hr.

Spore projection in Nigrospora

Webster (1952) has described violent spore discharge in *Nigrospora sphaerica*. Projection occurs under conditions of decreasing vapour pressure and appears to be due to the discharge of liquid through a fine orifice at the apex of a specialized conidiophore cell.

Colonies of *Nigrospora* occurring on decaying banana leaves were examined to discover whether violent spore discharge occurs on this substratum. Decaying leaf material was incubated for 2 days in a damp chamber to encourage spore formation. Thin strips of epidermal tissue were then rapidly transferred to the stage of a low-

power binocular microscope. Within a few seconds, presumably as the tissues dried out, spores were shot away from the substratum; no discharge occurred when the strips of tissue were examined while inside damp Petri dishes. Light did not affect the discharge process. Thus Webster's (1952) findings were confirmed.

DISCUSSION

It is clear that *Nigrospora* is a common component of the air-spores in Jamaican banana plantations. The fungus exhibits regular diurnal periodicity similar to that in *Nigrospora sphaerica* in Nigeria (Cammack, 1955), *Deighthoniella torulosa* in Jamaica (Meredith, 1961c) and *Phytophthora infestans* and *Polythrincium trifolii* in England (Hirst, 1953). Hirst (1953) suggested that this type of periodicity might be due to the existence of a definite discharge mechanism operating each day under conditions of decreasing vapour pressure. Observations reported here, and earlier ones of Webster (1952) and Cammack (1955), support this suggestion in the case of *Nigrospora*.

Rain and under-tree irrigation commonly resulted in large increases in concentration of *Nigrospora* spores. This was probably a result of increased sporulation of the fungus after wetting of the spore-bearing substratum, namely banana debris. November's data were exceptional in that there was no response to rainfall. An explanation for this must await further investigation into the ecology of *Nigrospora* on banana debris.

Since *Nigrospora* is not responsible for diseases of economic importance in Jamaica, the results presented here have little apparent practical value to the local banana industry. However in Australia, where 'squitter' disease (*Nigrospora sphaerica*) often assumes serious proportions, similar aerobiological studies may contribute to a better understanding of the epidemiology of disease.

Grateful acknowledgement is made to the United Fruit Co., Jamaica, for granting access to their plantations, and to Miss B. I. McLean, Miss V. E. Green and Mr R. R. Chen for valuable technical assistance.

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Nodule Bacteria Associated with the Indigenous Leguminosae of South-Western Australia

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(Received 22 April 1961)

SUMMARY

Root-nodule bacteria (rhizobia) isolated in pure culture from legumes indigenous to south-western Australia were examined for cultural and infective characters. All isolated strains had cultural characters consistent with the slow-growing bacteria of the lupin or soybean type. None of the strains nodulated peas, clover or medic, but hosts from the other four recognized cross-inoculation groups were nodulated. *Lupinus digitatus*, *Glycine hispida*, *Vigna sinensis* and *Phaseolus vulgaris* formed a natural grouping on the basis of susceptibility to nodulation by the native strains. Within the narrower host range of the genus *Lupinus* it was found that *L. digitatus*, *L. albus* and *L. pilosus* grouped together on susceptibility to nodulation, whereas *L. luteus*, *L. angustifolius* and *Ornithopus sativus* were not nodulated by any of the sample strains. The use of this geographically isolated sample of rhizobia has further demonstrated the weakness of the present scheme of classification of the nodule bacteria, which is based on the infective character of the bacteria. A taxonomic system based on Adansonian principles could be applied to the classification of the rhizobia, and a procedure for attempting this is outlined.

INTRODUCTION

The genus *Rhizobium* comprises a group of organisms characterized by their ability to induce the formation of nodules on the roots of legumes. Allen & Allen (1950) stated '... the ability to invade the roots of leguminous plants and stimulate the production of nodules... is the sole criterion for the existence of the genus *Rhizobium*'. Bacteria thus grouped on the feature of legume root nodulation are subgrouped on the range and type of host plants infected. *Bergey's Manual* (1957) accords six of these subgroups specific status but appends a note that some of them may be related. While this classification is generally recognized as imperfect, it persists in the absence of a better system. The present paper reports some results from a survey of the rhizobia associated with indigenous legumes of the botanical South-West Province of Western Australia (Lange, 1960). The province itself is about 100,000 square miles in area. Its climate was discussed by Gentili (1946), its geomorphology by Jutson (1950), soils by Teakle (1938) and the indigenous vegetation by Diels (1906) and Gardner (1942). Indigenous Leguminosae total over 400 species in about 35 genera of the Caesalpiniaceae, Mimosaceae and Papilionaceae. A great many of the species are endemic to the area. Nearly half are species of

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Acacia; the remainder are mostly papilionaceous, principally in genera of the tribe Podalyrieae. The incidence of nodulation in these indigenous legumes has already been tabulated (Lange, 1959).

METHODS

Over a 2-year period, 7000 miles of traverses were made throughout the study area and the vegetation examined at intervals, usually of only a few miles. Leguminous species were collected for identification and examined for root-nodulation. Species representative of the indigenous Leguminosae were selected from those found to be nodulated. Nodules were removed from these plants in the field, and desiccated in tubes over anhydrous CaCl_2 (Dr D. O. Norris, personal communication). In the laboratory the nodules were reconstituted by soaking in sterile water. They were then surface-disinfected by immersion for 15 min. in a mixture of equal volumes of H_2O_2 (100 vol.) and absolute ethanol, washed and macerated. Samples were streaked on plates of yeast extract + mannitol agar and incubated at 27° .

One hundred and sixty-three strains of presumptive rhizobia were isolated in pure culture. Eighty-five of these isolates from 83 host species in 24 legume genera were selected as a fair sample of the *Rhizobium* population associated with indigenous Leguminosae in the area (see Table 1).

Two series of tests were applied to the sample strains.

Tests of cultural characters

Colony characters. Visible characteristics of colonies on isolation plates and in pure culture were recorded. Strains of known performance from *Glycine*, *Lathyrus*, *Trifolium*, *Medicago*, *Phaseolus* and *Lupinus* were included in all tests to allow direct comparisons to be made.

Morphology and staining. Preparations from 6-day-old cultures were stained by Gram's method and with carbol rose bengal and examined.

Calcium glycerophosphate medium. Strains were streaked on slopes of calcium glycerophosphate agar (Hofer, 1941), incubated for 3 weeks at 27° and observed for browning of the medium.

Litmus milk. Strains were inoculated into sterile litmus milk, incubated for 4 weeks at 27° and observed for pH change and for serum zone formation.

Colony growth rate. Strains were dispersed in sterile water and dilution series made. Drops of appropriate dilutions were spread over yeast extract + mannitol agar plates to yield ten to thirty well-isolated colonies. Each plate was incubated at 27° , inspected daily, and the times for half of the colonies in sector areas to reach diameters of 1 mm. recorded.

Reactions on sugar. Cultures were inoculated on to nitrate agar medium (Baldwin & Fred, 1927) containing either arabinose, rhamnose, xylose, maltose or sucrose, and pH changes observed after incubation for 5 weeks at 27° .

Tests of infectiveness on legumes

Each of the 85 sample strains was further investigated in a series of glasshouse experiments to determine its infectiveness on the following legumes: *Pisum sativum* L., *Lathyrus cicera* L., *Lens esculenta* Moench., *Vicia sativa* L., *Lupinus angustifolius* L., *L. luteus*, L., *L. nanus* Dougl., *L. digitatus* Forsk., *L. albus* L., *L. pilosus* Murr.,

L. villosus Willd., *L. subcarneus* Hook., *Ornithopus sativus* Brot., *O. compressus* L., *Medicago tribuloides* Desr., *Trifolium subterraneum* L., *Vigna sinensis* Engl., *Phaseolus vulgaris* L., *P. lathyroides* L., and *Glycine hispida* Maxim. These include hosts from the seven major cross-inoculation groups. The eight *Lupinus* species were included since it had been indicated earlier that lupins may be differentially susceptible to native rhizobia in the area (Adams & Riches, 1930; Lange & Parker, 1960). The following conditions were imposed during the course of the experiments.

(1) Strains known to be infective on the hosts were included. These invariably nodulated the host legume under the conditions of experiment. This ensured that experimental conditions did not restrict nodulation of the plant.

(2) Experiments were terminated when the control nodulation was fully established.

(3) Control uninoculated plants were included at a frequency of 20 % of inoculated plants.

(4) Conditions for plant growth were kept as close to optimal as possible.

Experiments were conducted in closed glasshouse rooms with adequate sunlight and pressurized by air coolers delivering washed air. The rooms were sprayed down completely with insecticidal, fungicidal and bactericidal sprays, and with paraffin oil emulsion (25 % paraffin oil). Dust immobilization was maintained by further spraying with paraffin oil emulsion containing antibacterial substances. Large plants were grown in open sand-filled porous drainage pots spaced on slatted bench tops over an area heavily coated with paraffin oil emulsion. Pots were dressed with a nitrogen-deficient nutrient solution providing major and minor elements. Superphosphate was mixed with the sand separately at the rate of 300 p.p.m. as the fertilizer. *Trifolium* and *Medicago* plants were grown in agar tubes after the method of Chen & Thornton (1940).

Pots were brought to field capacity, wrapped, stacked in a bin and subjected to a continuous flow of steam for 1 hr.; this raised the temperature of the sand to at least 90°. Seeds were disinfected by immersion for 10–20 min. in a mixture of equal volumes of 100 vol. H₂O₂ and absolute ethanol, followed by washing in sterile water. The seeds were then germinated and planted under sterile conditions. Inoculum was applied direct to the seed coat, and pots were watered with sterile water. The tests were completed in a total of twelve experiments, each of factorial design: host species × sample strains × a minimum of four replications.

Root systems were washed out usually about 28 days after germination and floated in water for examination. In one experiment nodulation occurred in some of the uninoculated control plants; this experiment was repeated. In the experiments reported here the control of nodulation was absolute.

RESULTS

Cultural characters

All 85 bacterial strains originated from within the tissues of sound legume root-nodules and grew as aerobic heterotrophs. Colonies after 8 days were punctiform, rarely exceeding 1 mm. diam., and were opaque or rarely translucent, whitish, and gummy. No isolated colony exceeded punctiform size within 5 days, and all strains were therefore slow-growing compared to the known strains of *Rhizobium meliloti*,

354 Table 1. *The infectiveness of 85 strains of nodule bacteria on 9 legume test hosts*

+, Nodulation on all plant replications; ±, nodulation on some but not all plants; —, complete absence of nodulation.

Host of isolation	Strain no.	Host nodulated							Phaseolus lathyroides	P. vulgaris	Vicia sativa
		Glycine hispida	Lupinus albus	L. digitatus	L. pilosus	L. subcar-nosus	L. vil-losus				
<i>Acacia acuminata</i> Benth.	3	±	±	±	—	—	—	+	±	—	
<i>ericifolia</i> Benth.	54	—	—	+	—	—	—	+	±	—	
<i>stenoptera</i> Benth.	65	+	±	+	±	—	—	+	+	—	
<i>erinacea</i> Benth.	48	—	±	±	—	—	—	±	±	—	
<i>extensa</i> Lindl.	121	±	±	+	±	—	—	+	±	—	
<i>Drummondii</i> Lindl.	29	+	±	±	—	—	—	±	±	—	
<i>myrtifolia</i> Willd.	113	—	—	+	—	—	—	+	±	—	
<i>cyanophylla</i> Lindl.	20	—	±	+	+	—	—	±	±	—	
<i>hastulata</i> Smith	114	+	±	+	—	—	—	±	—	—	
<i>horridula</i> Meissn.	85	+	—	+	—	—	—	+	±	—	
<i>nervosa</i> D.C.	115	—	±	+	±	—	—	+	+	—	
<i>volubilis</i> F.v.M.	46	+	±	+	±	—	—	—	±	—	
<i>pulchella</i> R.Br.	64	—	±	+	±	—	—	—	±	—	
<i>diptera</i> Lindl.	14	+	±	+	—	—	—	+	±	—	
<i>Albizzia distachya</i> (Vent.) Macbride	97	+	—	+	—	—	—	+	±	—	
<i>Aotus villosa</i> Sm.	134	—	±	+	—	—	±	+	±	—	
<i>Preissii</i> Meissn.	126	+	±	+	±	—	—	+	±	—	
<i>Tietkinsii</i> F.v.M.	132	—	±	—	±	—	—	—	±	—	
<i>Bossiaea Presii</i> Meissn.	92	+	—	+	±	—	—	+	±	—	
<i>Laidlawiana</i> Tovey et Morris	96	—	±	—	—	—	—	—	—	—	
<i>Webbii</i> F.v.M.	110	—	±	+	±	—	±	±	±	—	
<i>dentata</i> Benth.	107	±	±	±	—	—	—	±	±	—	
<i>aquifolium</i> Benth.	95	±	—	—	—	—	—	+	±	—	
<i>eriocarpa</i> (R.Br.) Benth.	88	—	—	+	—	—	—	±	+	—	
<i>linophylla</i> R.Br.	99	—	—	—	±	—	—	±	±	—	
<i>Brachysema lanceolatum</i> Meissn.	91	±	±	+	±	±	—	+	—	—	
<i>sericeum</i> (Sm.) Domin.	117	+	—	—	—	—	—	+	±	—	
<i>praemorsum</i> Meissn.	124	—	—	—	—	—	—	—	—	—	
<i>Chorizema Dicksonii</i> Grah.	90	—	±	+	±	—	—	+	+	—	
<i>ilicifolium</i> Labill.	123	—	—	±	—	—	±	—	±	—	
<i>reticulatum</i> Meissn.	122	+	+	+	—	—	—	+	±	—	
<i>aciculare</i> (D.C.) C.A. Gardn.	120	+	—	±	+	—	—	—	+	—	
<i>cytisoides</i> Turcz.	119	+	—	+	—	—	—	+	±	—	
<i>ericifolium</i> Meissn.	52	+	+	+	±	—	—	+	+	—	
<i>Davesia hakeoides</i> Meissn.	57	±	—	±	—	—	—	—	±	—	
<i>brevifolia</i> Lindl.	101	±	—	±	—	—	—	—	±	—	
<i>pectinata</i> Lindl.	68	—	±	—	±	—	—	—	±	—	
<i>sphylla</i> (F.v.M.) Benth.	112	—	+	—	+	—	—	—	—	—	
<i>incrassata</i> Sm.	163	—	—	—	—	—	—	±	+	—	
<i>Dillwynia uncinata</i> (Turcz.) C.A. Gardn.	98	±	±	±	±	—	±	±	—	—	
<i>Euchilopsis linearis</i> (Benth.) F.v.M.	62	—	—	+	—	—	—	—	+	—	

		Host nodulated								
Host of isolation	Strain no.	<i>Gly- cine hispida</i>	<i>Lupi- nus albus</i>	<i>L. digi- tatus</i>	<i>L. pilosus</i>	<i>L. subcar- nosus</i>	<i>L. vil- losus</i>	<i>Phase- olus lathy- roides</i>	<i>P. vul- garis</i>	<i>Vigna sin- ensis</i>
<i>Eutaxia epacridioides</i> Meissn.	87	+	—	+	—	—	—	+	+	+
<i>virgata</i> Benth.	105	—	±	+	—	—	—	+	+	+
<i>densifolia</i> Turcz.	137	—	—	—	±	—	—	±	+	±
<i>Gastrolobium villosum</i> Benth.	106	—	—	+	—	—	—	±	±	+
<i>spinosum</i> Benth.	61	—	+	+	±	—	—	+	—	+
<i>obovatum</i> Benth.	32	—	±	±	—	—	—	±	±	+
<i>trilobum</i> Benth.	100	+	—	+	—	—	—	+	+	+
<i>Gompholobium marginatum</i> R.Br.	60	+	—	+	—	—	—	±	±	+
<i>venustum</i> R.Br.	66	—	—	+	—	—	—	—	—	+
<i>polymorphum</i> R.Br.	111	+	—	+	—	—	—	+	±	+
<i>Knightianum</i> Lindl.	108	±	+	+	+	—	—	—	+	—
<i>tomentosum</i> Labill.	104	±	±	+	—	—	—	+	±	+
<i>Hardenbergia Comptoniana</i> (Andr.) Benth.	19	+	±	+	±	—	—	—	+	+
<i>Hovea elliptica</i> D.C.	109	—	—	±	—	—	—	±	±	+
<i>trisperma</i> Benth.	15	+	±	+	—	—	—	+	+	+
<i>chorizemifolia</i> D.C.	26	+	±	+	±	—	—	—	—	+
<i>pungens</i> Benth.	118	+	±	+	±	—	—	+	±	+
<i>Isotropis cuneifolia</i> (Sm.) Domin.	16	+	±	+	±	—	—	—	+	+
<i>Jacksonia hakeoides</i> Meissn.	69	+	—	±	—	—	±	±	±	+
<i>spinosa</i> (Labill.) R.Br.	102	+	±	+	—	—	—	+	±	+
<i>horrida</i> D.C.	33	—	±	±	—	—	—	±	±	±
<i>furcellata</i> (Bonpl.) D.C.	125	+	±	+	±	—	—	+	±	+
<i>Kennedyia eximia</i> Lindl.	73	+	—	+	—	—	—	+	+	+
<i>coccinea</i> Vent.	77	+	±	+	—	—	—	+	+	+
<i>Stirlingii</i> Lindl.	78	+	±	+	±	—	—	+	±	+
<i>prostrata</i> R.Br.	2	+	±	+	—	—	—	+	±	+
<i>Stirlingii</i> Lindl.	76	+	—	+	—	—	—	+	+	+
<i>Latrobea hirtella</i> (Turcz.)	133	+	±	±	—	—	—	+	±	+
<i>Mirbelia floribunda</i> Benth.	89	±	±	+	—	—	—	+	±	+
<i>spinosa</i> Benth.	127	+	±	±	±	—	—	+	±	+
<i>dilatata</i> R.Br.	86	—	—	±	—	—	—	+	±	+
<i>Oxylobium parviflorum</i> Benth.	27	+	±	+	—	—	—	+	±	+
<i>lanceolatum</i> Benth.	130	±	—	+	—	—	—	+	+	+
<i>capitatum</i> Benth.	17	+	—	+	±	—	—	+	+	+
<i>atropurpureum</i> Turcz.	141	±	—	+	—	—	—	—	+	+
<i>reticulatum</i> Meissn.	81	—	—	+	—	—	—	+	±	+
<i>Pultenaea reticulata</i> (Sm.) Benth.	84	±	—	+	—	—	—	+	+	+
<i>strobilifera</i> Meissn.	85	+	—	—	±	—	—	+	+	+
<i>ochreatea</i> Meissn.	94	+	±	—	—	—	—	±	±	±
<i>Sphaerolobium alatum</i> Benth.	59	—	—	+	—	—	—	+	+	+
<i>medium</i> R.Br.	128	+	±	—	—	—	—	+	±	+
<i>grandiflorum</i> R.Br.	116	—	—	—	—	—	—	±	±	+
<i>Templetonia retusa</i> R.Br.	21	—	—	+	+	—	—	+	±	+
<i>Viminaria denudata</i> (Sm.)	93	+	+	+	—	—	—	+	±	+

R. trifolii and *R. leguminosarum* which were included. All strains were Gram-negative. None exhibited spores or morphologically distinctive forms, but ranged from coccobacilli to more or less straight bacilli from 0.5 to 4.5 μ by 0.5 μ . On calcium glycerophosphate agar all failed to cause browning. In litmus milk, all strains produced an alkaline reaction without appreciable dye reduction. None produced a serum zone. All 85 strains produced acid on arabinose and xylose, an acid reaction or no change of pH value on rhamnose, and alkaline reactions on maltose and sucrose.

Infectiveness

Under the conditions of the experiments, none of the 85 strains nodulated *Pisum sativum*, *Lathyrus cicera*, *Lens esculenta*, *Vicia sativa*, *Lupinus angustifolius*, *L. luteus*, *L. nanus*, *Ornithopus sativus*, *O. compressus*, *Medicago tribuloides*, or *Trifolium subterraneum*. The following plant species were nodulated by sample strains: *Vigna sinensis* (79 strains); *Phaseolus vulgaris* (76); *Lupinus digitatus* (71); *Phaseolus lathyroides* (67); *Glycine hispida* (53); *Lupinus albus* (47); *L. pilosus* (31); *L. villosus* (5) and *L. subcarnosus* (1). The details of their infective performance on these species are presented in Table 1.

DISCUSSION

Breaches of the cross-inoculation boundaries between the soybean and the cowpea (Leonard, 1923), and between lupins, soybeans and cowpea (Bushnell & Sarles, 1937) have been reported before. The results submitted here further illustrate the deficiencies of the cross-inoculation-group concept when used to delineate species of *Rhizobium*. The bacterial strains used in this study were selected as representative of the microsymbionts from indigenous legumes in the study area. When compared with recognized *Rhizobium* species on the basis of their *in vitro* characters, all of them showed the characteristics of the slow-growing species *Rhizobium japonicum*, or of the cowpea group of rhizobia.

The range of infective performance exhibited by the sample strains extended to hosts from four of the recognized cross-inoculation groups, namely, the cowpea, lupin, soybean and bean groups. However, infection across the boundaries of these four groups was prevalent to the extent of being typical rather than exceptional. Thus of the 85 strains examined

- 45 strains nodulated hosts in 4 cross-inoculation groups;
- 31 strains nodulated hosts in 3 cross-inoculation groups;
- 6 strains nodulated hosts in 2 cross-inoculation groups;
- 3 strains nodulated hosts in 1 cross-inoculation group.

On the evidence it is impossible to place these strains in specific cross-inoculation groups, or to assign species designations to them on the basis of the accepted system. Distinctly different host groupings are apparent. For example, *Lupinus digitatus*, *Glycine hispida*, *Vigna sinensis* and *Phaseolus vulgaris* group together on susceptibility to infection by the sample strains, even though these hosts are from four different cross-inoculation groups. Similarly, *L. digitatus*, *L. albus* and *L. pilosus* group with each other on susceptibility, but do not group at all with their accepted cross-inoculation associates *L. angustifolius*, *L. luteus* and *Ornithopus sativus*.

Three *Rhizobium* species were not involved at all. None of the data implicated the clover, the medic or the pea cross-inoculation groups in any way, although

evidence from other sources (Kleczkowska, Nutman & Bond, 1944; Norris, 1959) shows that the pea and clover groups are not as discrete as they were once regarded.

Past studies of root-nodule bacteria show preoccupation with symbiont infectiveness patterns because of the economic importance attached to them. For the same reason, there has been a further preoccupation with agronomic legumes. This bias has dominated microsymbiont classification. In the period around 1920 there were two conflicting approaches. On the one hand, an expedient classification of the bacteria was derived based solely on their segregations in symbiotic infectiveness patterns (Garman & Didlake, 1914). On the other hand, the practice of classifying strains on the basis of symbiotic performance alone was rejected by Löhnis & Hansen (1921). Literature published over the succeeding period recorded the continued dominance and development of the former approach (Fred, Baldwin & McCoy, 1932). A species of *Rhizobium* was recognized as containing those microsymbionts which would nodulate a particular group of legumes. Other characters of the bacteria were not accorded equivalent taxonomic weight. Subsequently, patterns of symbiotic infectiveness were demonstrated to be less discrete (Leonard, 1923; Sears & Clark, 1930; Raju, 1936; Bushnell & Sarles, 1937). Interrelationships led some authors to suggest consolidations of groups and of species (Walker & Brown, 1935), whilst others suggested retention of the old groups with provision for special cases (Allen & Allen, 1936).

A second and very emphatic rejection of the cross-inoculation basis of strain classification was made by Wilson (1939) who produced substantial data to support his contentions. Further demonstrations of the inadequacies of the cross-inoculation groups continued (Appleman & Sears, 1942; Johnson & Allen, 1952; Ishizawa, 1954; Bowen, 1960). Recently a third rejection of the cross-inoculation-group concept was made. Norris (1956) considered that there was no satisfactory known basis for the subdivision of the rhizobia into species. He suggested the use of a symbiotic rating which would indicate the relative affinities of a particular strain for hosts within the three subfamilies of the Leguminosae.

The suggested modifications all continue to classify the nodule bacteria on the basis of their infective performance, excluding comparisons with bacteria which cannot produce nodules on the roots of a legume. This is clearly unsatisfactory. To be effective as a classification of bacteria, a system should be erected on non-biased over-all similarities. The Adansonian classification proposed by Sneath (1957) fulfils these requirements. The classification of the root nodule bacteria might thus be resolved by research within the following stages:

- (1) Collection of types (or neotypes) of all recognized *Rhizobium* species.
- (2) Collection from botanically representative and geographically diverse sources the widest sample of legume root-nodule bacteria possible within research resources.
- (3) Collection of types and representatives of possibly related bacteria, e.g. *Agrobacterium* (Hofer, 1941; Bonnier, 1953); *Bacillus* (Bisset, 1952, 1959); *Beijerinckia* (Derx, 1953).
- (4) Derivation of a range of tests by which all these bacteria may be described.
- (5) Application of the tests, and classification of all strains on the results, by Sneath's (1957) method.

By thus relating a full range of root-nodule bacteria to each other and to other bacteria, their over-all similarities and groupings should become evident, and the

genus *Rhizobium* with its component species could be retained or rejected. Application of an Adansonian classification to the strains used in this study would be premature, since both the strain sample and the test range are not adequate for the derivation of a generalized classification.

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The Occurrence of Polythionates as Intermediates in the Metabolism of Thiosulphate by the Thiobacilli

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SUMMARY

Further evidence for the inclusion of polythionates in the pathway of thiosulphate oxidation by members of the genus *Thiobacillus* is presented. Manometric experiments showed tetrathionate oxidation to be a stage in thiosulphate oxidation by suspensions of *T. thioparus* and *T. thiooxydans*. *Thiobacillus thioparus* accumulated a sufficient concentration of polythionate in the medium to allow chromatograms to be prepared, and the type of polythionate accumulated was influenced by the ratio between the sodium and potassium ions in the medium. The restriction of the oxidation of tetrathionate at the lower concentrations of K^+ may be due to a restriction in the entry of phosphate into the cell during growth; this is discussed.

INTRODUCTION

The first report of polythionate accumulation by a *Thiobacillus* sp. growing in a thiosulphate medium was made by Nathansohn (1902) when he described the accumulation of an incompletely oxidized sulphur compound which did not react with iodine, but which was oxidized to sulphate by bromine. Since that time there have been several attempts to show the accumulation of polythionates by these organisms. Starkey (1935) was unable to detect the formation of polythionates by pure cultures of *T. thioparus* and *T. novellus*. He showed the formation of tetrathionate in cultures of *T. trautweinii*, which he considered was a heterotrophic organism and which has since been reported to be facultatively autotrophic (Parker & Prisk, 1953). Skarzynski & Szczepkowski (1959), by using a chromatographic method, were unable to detect the presence of polythionates in cultures of *T. thioparus*; they concluded that polythionates were not concerned in thiosulphate oxidation. The manometric experiments of Vishniac (1952) suggested that tetrathionate and trithionate were intermediates, whilst Trudinger (1959) showed the incorporation of radioactive ^{35}S into tetrathionate and trithionate, and subsequently into a compound which he was unable to identify. Parker & Prisk (1953) showed the formation of tetrathionate by some *Thiobacillus* spp., but not by *T. thioparus*, and Pratt (1958) showed the formation of polythionate by the strains of *T. thioparus* and *T. thiooxydans* used in this work.

METHODS

Organisms

Thiobacillus thiooxydans. A strain derived from a single rod obtained from a crude culture oxidising thiocyanate.

Thiobacillus thioparus. The original culture was obtained from the National

Collection of Industrial Bacteria—NCIB 8370—described as 'Starkey's original non-motile strain'. This culture was purified by the isolation of a single colony on a thiosulphate agar plate.

Both these organisms have been maintained on thiosulphate agar slopes with weekly subculturing.

Thiosulphate medium

A solution containing: 10.0 g. $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$; 3.5 g. KH_2PO_4 ; 1.4 g. KOH; 0.1 g. NH_4Cl ; in 1 l. glass distilled water was autoclaved at 120° for 15 min., and after cooling, 10 ml. each of the sterile salt solutions B and C were added. Salt solution B contained 1.0 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.2 g. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$; 0.2 g. FeCl_3 ; 3 ml. concentrated hydrochloric acid; glass distilled water to 100 ml. Salt solution C was a solution (1%, w/v) of calcium chloride in glass distilled water. Both salt solutions were sterilized by autoclaving at 120° for 15 min. The complete medium was at pH 7. Solid media were prepared by adding 4% (w/v) washed agar to thiosulphate medium prepared at twice the concentration shown above to give a final agar concentration of 2% (w/v).

Suspensions of organism

The organisms were grown in 2×5 l. batches aerated with air containing 5% (w/v) carbon dioxide at 30° – 32° for 3–4 days. Harvesting was carried out with a de Laval centrifugal separator and a refrigerated M.S.E. centrifuge. All the equipment coming into contact with the organisms during harvesting was sterilized before use. Ten l. of thiosulphate medium produced about 1.0 g. wet wt. organism which was suspended in 20 ml. of M/15 phosphate buffer (pH 7.0) made from Na_2HPO_4 and KH_2PO_4 .

Manometry

Manometric experiments were carried out in a conventional Warburg apparatus under aerobic conditions at 30° . Carbon dioxide was absorbed with filter paper soaked in 0.2 ml. 20% (w/v) KOH solution in the centre well. Substrate (0.5 ml. M/50) was put in the side arm, 1.0 ml. of cell suspension in the main compartment with M/15 phosphate buffer (pH 7.0), to bring the total volume of the flask contents to 3.0 ml. The flasks and manometers were equilibrated in the bath for 15 min. before the reaction was started.

Estimation of polythionate

The method used was that described by Starkey (1934) which depends on the conversion of polythionates to thiosulphate and sulphite when heated with KOH.

Chromatography

Identification of the accumulated polythionates was attempted by ascending chromatography, with the solvent isopropanol + acetone + water + potassium acetate (50 + 20 + 30 + 2 g.) described by Pollard, McOmie & Jones (1955). Portions (10 ml.) of culture filtrate was freeze-dried, taken up in 0.5 ml. distilled water, and spotted on acid-washed Whatman No. 1 chromatography paper. After running for 5 hr.

the chromatograms were dried, sprayed with 0.5 N-silver nitrate, and excess silver removed by successive washings with water, 50 % (w/v) sodium thiosulphate solution, and water. The markers used for chromatography were sodium thiosulphate (Analar) and the potassium salts of tetrathionate and trithionate prepared by the methods described by Stamm, Goehring & Feldmann (1942).

RESULTS

Manometric experiments

Manometric experiments on the oxidation of thiosulphate showed similar results for suspensions of both organisms. The change in slope of the thiosulphate oxidation curve reported by Vishniac (1952) was observed, although in our experiments this change occurred after an oxygen consumption in excess of the 56 μ l. reported by

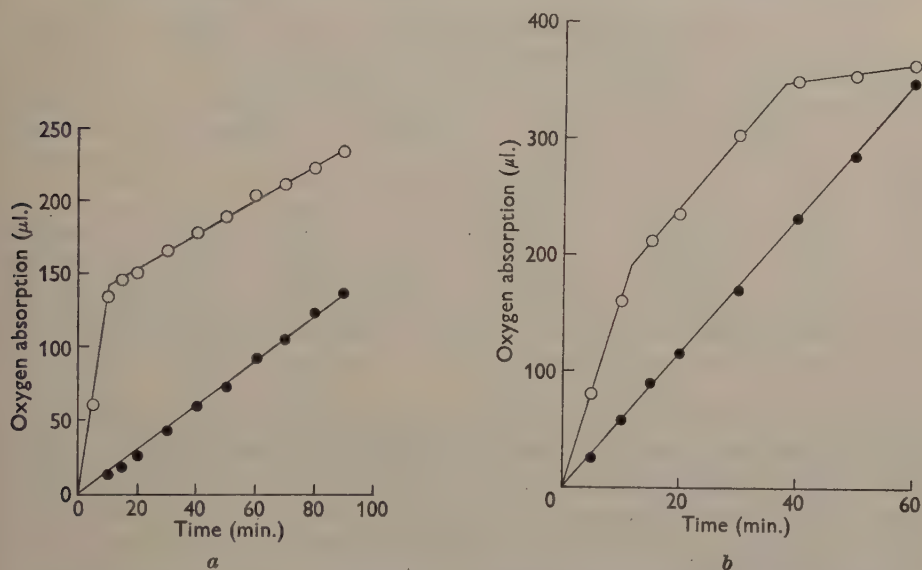


Fig. 1. (a) Oxidation of thiosulphate \circ , and tetrathionate \bullet , by suspensions of *Thiobacillus thioparus*. (b) Oxidation of thiosulphate \circ , and tetrathionate \bullet , by cell suspensions of *Thiobacillus thiooxydans*.

Vishniac. Figures. 1a and b shows the rates of thiosulphate and tetrathionate oxidation by *Thiobacillus thioparus* and *T. thiooxydans*. We endeavoured to obtain evidence of the presence of tetrathionate at the time when the change in slope occurred. To stop the reaction, the reaction mixture from the flasks was tipped into ethanol, the organisms centrifuged down and the supernatant fluid freeze-dried. Chromatograms showed no trace of polythionate.

Accumulation of polythionates

In the standard medium cultures of *Thiobacillus thioparus* accumulated considerable amounts of polythionate in the medium during growth. The chromatogram obtained from cultures of *T. thioparus* grown at 32° in static culture for 67 hr. showed a spot which corresponded to the trithionate marker. With further incubation

another spot running in advance of the tetrathionate marker appeared which we think may be pentathionate. Our attempts to prepare a pentathionate standard with which to make a direct comparison were unsuccessful.

In their review on the thiobacilli, Vishniac & Santer (1957) gave details of a requirement for either phosphate or arsenate to allow complete oxidation of thiosulphate to sulphate by suspensions of *Thiobacillus thioparus*. Experiments were therefore carried out with media containing higher concentrations of sodium potassium phosphate buffer than usual in an attempt to suppress the accumulation of polythionate in cultures of *T. thioparus*. The results of such an experiment are shown in Table 1. The phosphate concentrations used were of the same order as those described by Vishniac & Santer (1957) for their manometric experiments.

Table 1. *The effect of phosphate concentrations upon the utilization of thiosulphate and the accumulation of polythionate (S_nO₆) by Thiobacillus thioparus*

Cultures were grown in 250 ml. conical flasks containing 50 ml. of medium. Each culture was inoculated with 0.5 ml. of an aseptically filtered 4-day thiosulphate grown culture.

Time (hr.)	Phosphate concentration														
	M/10			M/20			M/30			M/100			M/250		
	S ₂ O ₃	S _n O ₆	pH	S ₂ O ₃	S _n O ₆	pH	S ₂ O ₃	S _n O ₆	pH	S ₂ O ₃	S _n O ₆	pH	S ₂ O ₃	S _n O ₆	pH
0	196	2	6.78	201	0	6.78	205	1	6.78	208	0	6.46	198	1	5.97
23	196	6	6.85	192	8	6.84	195	9	6.78	196	10	6.59	190	11	6.23
47	168	4	6.68	162	31	6.74	175	22	6.71	167	37	6.78	137	37	6.55
71	143	3	6.65	153	27	6.71	129	64	6.83	130	75	7.00	78	59	6.39
95	126	2	6.63	117	53	6.78	86	102	6.89	86	90	7.27	2	77	5.86
115	75	2	6.59	60	100	6.75	47	119	6.76	53	124	7.36	0	75	4.07
139.5	79	4.5	6.6	23	109	6.72	18	128	6.74	18	162	7.32	0	83	4.23

Polythionate accumulation was greatly decreased at the highest concentration but in this medium the thiosulphate was not completely utilized. Examination of the chromatograms from these cultures again showed two spots other than that for thiosulphate. The unknown component running in advance of tetrathionate again appeared, but in place of trithionate a spot corresponding to the tetrathionate marker was observed.

The effect of varying the ratio between sodium and potassium ions in the medium was examined. The method used for increasing the phosphate concentration of the medium in the previous experiment increased the ratio between the sodium and potassium ions in the medium. Thus the medium described in the methods section has a ratio of 0.93 g. ion sodium:1 g. ion potassium, whereas in the experimental cultures this ratio varied between 4.2:1 for M/10 phosphate and 21.4:1 for M/250 phosphate. It seemed possible that this increased ratio between the sodium and potassium ions in the medium might be responsible for the change in the type of polythionate accumulated by the organism. By the replacement of some of the potassium salts in the standard medium by the equivalent amounts of the sodium salts, media having ratios between the sodium and potassium ions of 2.4:1, 5.6:1, and 8.7:1 were prepared. The type of polythionate accumulated by the organism growing in these media was compared with that accumulated during growth on the standard medium. A chromatogram was obtained from a culture in a medium having a ratio between the sodium and potassium ions of 5.6:1, and was typical of the

chromatograms obtained from the other two media which had high ratios between the sodium and potassium ions. The chromatogram showed accumulation of tetrathionate instead of trithionate, and also the component which ran in front of tetrathionate.

Further experiments were made to investigate the effect of decreasing the ratio of sodium ion to potassium ion below 0.93:1. Substitution of part of the sodium thiosulphate by the equivalent amount of the potassium salt produced a series of media having ratios of 0.81:1, 0.57:1, and 0.45:1, between the sodium and potassium ions. The type of polythionate accumulated during the growth of *Thiobacillus thioparus* in these media was compared with that accumulated during growth in the standard medium. Typical chromatograms obtained from the cultures grown in the medium having a ratio of Na:K of 0.57:1 showed the accumulation of trithionate and the component which ran in advance of the tetrathionate marker.

DISCUSSION

Evidence has been presented for the inclusion of polythionates in the pathway of thiosulphate oxidation by the thiobacilli and supports that obtained by Vishniac (1952) and Trudinger (1959) that the initial stages in thiosulphate oxidation by the thiobacilli are:



Manometric experiments with suspensions of *Thiobacillus thioparus* and *T. thiocyanoxidans* showed both organisms to be capable of oxidising tetrathionate. The rate of oxidation of this substrate is similar to that shown by the second part of the thiosulphate oxidation curve (see Fig. 1*a* and *b*). These results are similar to those obtained with *T. thioparus* by Vishniac (1952), except that the change in slope occurred at an oxygen absorption in excess of the 56 μl . observed by Vishniac. Baalsrud & Baalsrud (1954) suggested that in the case of *T. denitrificans* such changes in the rate of thiosulphate oxidation were brought about by damage which the cells sustained when the pH value of the medium decreased during growth. Extending this view to *T. thioparus* and *T. thiocyanoxidans* it seems possible that the disparity between our results and those of Vishniac about the quantity of oxygen utilized before the change in rate of thiosulphate oxidation occurs was caused by this phenomenon.

Although we did not observe the presence of polythionates in the reaction vessels during manometric experiments, *Thiobacillus thioparus* was shown to accumulate considerable amounts of polythionate in the medium during growth on thiosulphate. Cultures grown in the standard thiosulphate medium were shown by chromatographic methods to accumulate trithionate and what may be pentathionate. The work of Vishniac & Santer (1957) showed a requirement by suspensions of *T. thioparus* for phosphate or arsenate to allow complete oxidation of thiosulphate. Increasing the phosphate concentration of the growth medium to M/10 resulted in a considerable suppression of polythionate accumulation. Further experiments which involved altering the ratio between the sodium and potassium ions in the medium indicated that this ratio was also of importance in the oxidation of thiosulphate by growing cultures.

The absorption of potassium in yeast, *Escherichia coli*, '*Bacterium lactis aerogenes*' and some marine bacteria occurs only in the presence of a substance which can pro-

duce energy; phosphate uptake by these species of organisms is stimulated by the presence of potassium, each K^+ ion being associated with the loss of a H^+ ion from the cell and each phosphate being associated with the gain of a H^+ ion by the cell (Rothstein, 1959). Comparable factors may well operate here, the lower concentrations of potassium in some of the media restricting the amount of phosphate entering the cell and thus preventing the further oxidation of tetrathionate.

Our initial experiments on the accumulation of polythionate in the medium were carried out with cultures of *Thiobacillus thiocyanoxidans* and *T. thioparus*; only very small amounts of polythionate were observed in the cultures of *T. thiocyanoxidans*.

From time to time there appear in the literature indications of more complex sulphur compounds as intermediates in the pathway of thiosulphate metabolism. Skarzynski & Szczepkowski (1959) suggested that polythionates were not concerned, and that the sulphur passed through organic intermediates. Santer *et al.* (1960) also suggested the existence of an organic intermediate, and Trudinger (1959) showed the incorporation of radioactive sulphur into an unknown compound after its incorporation into tetrathionate and trithionate. There seems little doubt that polythionates are concerned in thiosulphate metabolism, and perhaps the suggested organic intermediates are concerned in the unknown steps which convert trithionate to sulphate.

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On the Survival of Frozen Bacteria

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SUMMARY

Steadily growing *Aerobacter aerogenes* organisms were largely killed by slow freezing in buffer or by freeze drying. 100% survival was obtained after dropping bacteria suspended in 10% aqueous glycerol into liquid nitrogen and thawing. Ten per cent solutions of diethylene glycol, *i*-erythritol, glucose, sucrose or polyethylene glycol (MW = 10,000) protected equally well; the last three substances did not penetrate the cell cytoplasm. The most lethal medium was dilute NaCl; broth, water or a dilute salt mixture were moderately lethal. These findings are incompatible with the view that the lethal effects of freezing are connected with osmotic shock or that protection from freezing damage requires (*a*) penetration of the protective agent or (*b*) osmotic dehydration of the cytoplasm. Cells frozen and thawed, even with a protective agent, showed a lowered rate of glycerol oxidation and a higher death rate when starved at the optimal temperature and pH value for growth. The storage life of frozen organisms at -20° depended on the protective agent used; only glycerol permitted extended storage.

INTRODUCTION

Rapid chilling of microbial suspensions by dropping them into liquid nitrogen has been used by some workers as a means of disrupting the organisms. Moses (1955) obtained preparations of citric acid cycle enzymes from the mould *Zygorrhynchus moelleri* in this manner; Postgate (1960) used it to obtain a particulate sulphite reductase preparation from *Desulfovibrio desulfuricans*; Wade & Lovett (1961) extracted ribonucleic acid from *Escherichia coli* type B by such a procedure. Paradoxically, however, a considerable volume of reports exists demonstrating that bacteria survive chilling to very low temperatures. Earlier work was reviewed by Luyet & Gehenio (1940), who pointed out that much of the data were not quantitative, but who recorded figures as divergent as 11% kill with *E. coli* in broth frozen to -78° (Sanderson, 1925) to 99.98% kill of *Bacterium typhosum* (*Salmonella typhi*) in broth (Smith & Swingle, 1905) after freezing at -17.8° . Haines (1938) obtained only 5% killing of a staphylococcus by freezing in solid CO_2 and kills of other micro-organisms were small. Wieser & Osterud (1945) recorded modest kills of *E. coli* on freezing (15% at -2° , 50% at -5° or -10°); Straka & Stokes (1959) reported about 10% kills of *E. coli* and pseudomonads on freezing in beef broth; Wood & Rosenberg (1957) recorded 100% survival of yeast frozen in phosphate buffer at -60° . Mazur, Rhian & Mahlandt (1957) reported that slow chilling and rapid warming afforded maximum survival of *Pasteurella tularensis* frozen in 'gelatin saline' to temperatures between -10° and -70° . In all circumstances rather low survivals compared with unfrozen

controls were obtained, but Mazur (1960) mentioned an experiment in which the addition of sucrose or lactose (0.3M) permitted nearly 100 % survival after slow cooling and slow warming.

Harrison & Cerroni (1956) recorded very slight killing of *Microbacterium flavum* on repeated freezing to -22° and thawing; *Escherichia coli* showed much greater sensitivity though both species were about equally sensitive to mechanical damage. Major, McDougal & Harrison (1955) studied the survival of various bacteria stored frozen in broth at -22° and showed that in most instances the survival was longer the denser the initial population; their data did not include survivals measured immediately after freezing except in the case of *E. coli* frozen in distilled water, which then showed a small (about 10 %) kill and no population effect. Record & Taylor (1953) failed to observe a population effect with *E. coli* frozen in phosphate buffer at -78° and thawed at once (kill 40 %). Harrison (1955) showed that the population effect did not occur with *Lactobacillus fermenti* after a single freeze and thaw (about 90 % kill) but that it was manifest after repeated freezing and thawing with intervals of storage. Aeration influenced the resistance of *E. coli* to freezing in a complex manner. Harrison (1956) studied the influence of environment on freezing three bacterial species at -22° and showed that broth was a more lethal environment for freezing than was distilled water. Suspensions chilled to -22° but prevented from freezing with NaCl (4.6M) or glycerol (4.1M) had similar store lives to frozen suspensions.

Hollander & Nell (1954) showed that addition of 15 % glucose to broth permitted 100 % survival of *Escherichia coli*, *Diplococcus pneumoniae*, *Treponema pallidum* and probably of *Rhodospirillum rubrum*. They considered the protective effect of glycerol to be analogous to its action on bull spermatozoa (Polge, Smith & Parkes, 1949) or erythrocytes (Lovelock, 1953). Howard (1956) used a frozen glycerol broth according to Hollander & Nell's prescription to preserve cultures of bacteria; so did Tanguay (1959) and Floodgate & Hayes (1961). Fox & Hotchkiss (1957) used freezing in aqueous glycerol to preserve pneumococci in a receptive state for transforming deoxyribonucleic acid. None of these workers quoted quantitative data on survival after freezing. Squires & Hartsell (1955) reported some quantitative data on the survival of *E. coli* after freezing at -25° and storage at -9° ; as an additive 5 % glycerol was superior to gelatin and a vegetable oil for organisms frozen in a buffered broth. Quadling (1960) reported 100 % survival of *Xanthomonas phaseoli* on freezing in strong glycerol broth, though exposure of the organisms to this medium killed about 60 % of the population without freezing.

In the course of other studies we wished to preserve populations of steadily growing *Aerobacter aerogenes* while retaining as nearly as possible 100 % viability, and we performed a number of experiments on deep freezing. These are reported below, since they have some relevance to the question of freezing damage and were performed on a single culture of the same organism in a phase of growth analogous to the logarithmic phase, commonly associated with enhanced sensitivity to stresses of various kinds including freezing (Toyokama & Hollander, 1956); in addition they deal largely with survival in solutions of single pure substances.

METHODS

Aerobacter aerogenes, obtained from Professor Sir Cyril Hinshelwood's laboratory, had been maintained for some 18 months in continuous aerobic culture in a defined (0.2 %, w/v) glycerol medium analogous to that used for *Cloaca cloacae* by Herbert, Elsworth & Telling (1956). We shall describe the cultural conditions and history of the strain in more detail elsewhere; for present purposes it is sufficient to record that the dilution rate was 0.25 vol./hr., pH 7 ± 0.1 , 40°; growth limited by glycerol concentration to 1.05 ± 0.05 mg. dry wt. organism/ml. This corresponded to a viable count of about 2.4×10^9 organisms/ml., equal to the total count determined by a combination of microscopy and interferometry (Norris & Powell, 1961).

Viability was determined by slide culture at 37° (Postgate, Crumpton & Hunter, 1961) on the growth medium supplemented with yeast extract (Difco, 0.1 %, w/v), casein hydrolysate (Difco, 0.1 %, w/v), a tryptic meat broth (10 %, v/v) and set with agar (1.5 %, w/v). The nitrogenous supplements should have ensured growth of 'nutritionally injured' organisms of the type demonstrated by Straka & Stokes (1959). Before slide culture, frozen populations were thawed by diluting in 50 or 500 vol. saline phosphate buffer (9 parts 0.137 M-NaCl + 1 part of a 1:5 mixture of 0.066 M- KH_2PO_4 + 0.066 M- Na_2HPO_4 ; pH 7.40 ± 0.05) at room temperature. In one experiment death curves were measured in a saline 'tris' buffer (9 parts 0.137 M-NaCl + 1 part 0.048 M-2-amino-2-hydroxymethylpropane-1:3-diol ('tris') + 0.316 mM ethylenediaminetetra-acetic acid (EDTA); pH 7.00 ± 0.05). Reagents of analytical grade were used except that *i*-erythritol and D-glucose were 'bacteriological' sugars, diethylene glycol was a re-distilled (b.p. 135–137°) specimen kindly provided by Major L. H. Kent, polyethylene glycol was a well-dialysed, freeze-dried preparation of mean mol. wt. 10,000 kindly provided by Dr B. R. Record. Water was distilled and then de-ionized by treatment with a mixed bed ion exchange resin. Percentages recorded in this paper are w/v except in the cases of glycerol and diethylene glycol when they are v/v.

RESULTS

Sensitivity of the population to cold

Gram-negative bacteria in the logarithmic phase of growth are sometimes killed by cold shock even when no freezing takes place (Sherman & Cameron, 1934; Hegarty & Weeks, 1940). This phenomenon requires a 'toxic' suspending fluid of low osmotic pressure (Meynell, 1958) and is not shown by staphylococci (Gorrill & McNiel, 1960). The population of *Aerobacter aerogenes* used in this work did not show appreciable cold shock of this kind. Organisms harvested from the culture chamber, washed by centrifugation in 0.8 % NaCl and diluted into cold buffer showed 99 % viability in saline phosphate buffer at room temperature, 98 % at 4°, 94 % after squirting on to frozen buffer at its melting point. Suspensions were, however, killed when 10 ml. portions in tubes were frozen in solid CO_2 : the initial viability fell from 96 % to less than 1 % in these conditions. Use of *mist. desiccans* (Fry & Greaves, 1951) in place of buffer afforded no protection (93.8 % viable population fell to less than 0.3 % after freezing in *mist. desiccans* with solid CO_2). Effluent from the continuous culture freeze-dried without further treatment fell from about 98 to 5 %; centrifugation and resuspension in *mist. desiccans* provided no significant protection (viability fell to

6%). The low survivals obtained in these conditions are probably attributable to our use of organisms in what corresponds to a logarithmic phase of growth. Organisms re-suspended in the original medium (containing 0.2% glycerol) and freeze-dried had the marginally greater viability of 10%.

Protection from killing by freezing

A thick suspension (equiv. about 50 mg. dry wt. organism/ml.), resuspended in a salt mixture corresponding to the growth medium without glycerol, was allowed to drop from a pipette into liquid nitrogen, so that the suspension froze as discrete beads. These were allowed to thaw to room temperature and showed 35% viability. This observation suggested that deep freezing might provide a method of preserving the population, and the viability (in various suspending media) after deep freezing with liquid nitrogen was studied. Organisms were harvested from the continuous culture, centrifuged and made up as suspensions equivalent to 1 or 10 mg. dry wt. organism/ml. Within 5–15 min. these were frozen as beads of about 0.05 ml. by dropping from a pipette into liquid nitrogen. This procedure took 1–2 sec. The beads were then removed after 3 to 10 min. with cold forceps and thawed by dropping into 25 or 2.5 ml. saline phosphate buffer at room temperature. This procedure adjusted the population density to a value suitable for slide culture; thawing in these conditions took 2–3 sec.

Table 1 records a representative set of experiments. Suspension in 10% aqueous glycerol protected all the viable organisms from killing by freezing (1*b*; 2*a*, *b*, *c*); there was no population effect of the kind reported by Major *et al.* (1955) over a 100-fold range of suspension concentrations (1*c*, *d*; 2*a*, *b*, *c*). A tryptic broth of meat allowed considerable killing (4*f*); glycerol as prescribed by Hollander & Nell (1954) protected the organisms from the toxicity of broth (4*g*). Unlike the organisms studied by Major *et al.* (1955), Harrison (1956) and Clement (1961), considerable kills of our bacteria occurred on freezing in distilled water (1*a*); comparable kills were obtained when suspensions equivalent to 1 mg. dry wt. organism/ml. were frozen and thawed in (i) distilled water which had not been treated with resin (viability: 58%), (ii) water distilled twice, the second time from glass (viability: 67%), (iii) the distilled and resin-treated water usually used in this work (viability: 68%) or (iv) our local tap water (viability: 53%). Sodium EDTA (10 mM) had no significant effect on the kill (viability: 42% with EDTA, 49% without). These experiments make it unlikely that impurities in the distilled water or population effects contributed to the kill. Carry-over of salts in the washing procedure likewise did not account for the kill, since a suspension repeatedly centrifuged in distilled water gave 68, 47 and 48% viabilities after the first, second and third centrifuging; the unfrozen control was 98% viable after the third centrifuging.

NaCl solutions were the most lethal environments encountered and the kill after freezing and thawing was independent of the salt concentration over a wide range (5*a–e*). Buffered sodium chloride with 'tris' made little difference (5*f*), but phosphate had a marginal protective effect (5*g*) and probably accounts for the lowered kill in the substrate-free medium (1*c*, *d*) which was essentially a phosphate mixture with trace elements. Addition of glycerol to the medium afforded partial protection (1*e*). Harrison (1956) showed that glycerol protected organisms frozen in salt solutions.

Glycerol at 10 % also protected stationary phase organisms harvested from an agar slope (3*a*).

The glycerol concentration could be reduced to 2 %, but not below this value, without losing its protective effect. It could be replaced by 10 % aqueous solution of diethylene glycol (4*a*), glucose (4*b*), *i*-erythritol (4*e*), sucrose (4*c*) and a polyethylene glycol of mean molecular weight 10,000 (4*d*). Though some of these solutions had slight intrinsic toxicities, lowering the initial viability of the population from about 97 to about 90 %, the survivors were wholly protected from freezing. The molarities of these solutions were: glycerol, 1.08 M; diethylene glycol, 0.93 M; *i*-erythritol, 0.82 M; glucose, 0.55 M; sucrose, 0.29 M; polyethylene glycol, 0.01 M.

Table 1. *Viabilities of suspensions of Aerobacter aerogenes after a single freeze and thaw*

Aerobacter aerogenes growing at an imposed doubling time of 2.8 hr. in continuous culture were centrifuged, drained and re-suspended in the solutions indicated at about equiv. 10 mg. dry wt. organism/ml. in experiments 1 and 2, equiv. 1 mg. organism/ml. in experiments 3, 4 and 5. Drops (about 0.05 ml.) of suspension were frozen in liquid nitrogen diluted in buffered saline and the % viable organism determined by slide culture.

Experiment	Medium	Viability (%)	
		Control	Frozen
1 <i>a</i>	Distilled water	97	63
1 <i>b</i>	Aqueous 10 % glycerol	97	96
1 <i>c</i>	Basal medium without carbon source	97	39
1 <i>d</i>	As 1 <i>c</i> but equiv. 1 mg. dry wt. organism/ml.	97	32
1 <i>e</i>	Basal medium + 10 % glycerol	97	70
2 <i>a</i>	10 % glycerol	97	96
2 <i>b</i>	As 2 <i>a</i> , but equiv. 1 mg. dry wt. organism/ml.	97	95
2 <i>c</i>	As 2 <i>a</i> but 0.1 mg. organism/ml.	97	95
3 <i>a</i>	10 % glycerol; stationary phase organisms from agar slope	90	89
4 <i>a</i>	10 % diethylene glycol	97	95
4 <i>b</i>	10 % glucose	91	88
4 <i>c</i>	10 % sucrose	98	95
4 <i>d</i>	10 % polyethylene glycol, mol. wt. about 10,000	92	89
4 <i>e</i>	10 % <i>i</i> -erythritol	97	95
4 <i>f</i>	Tryptic meat broth	93	48
4 <i>g</i>	As 4 <i>f</i> + 15 % glycerol	92	91
5 <i>a</i>	M-NaCl (5.6 %)	95	12
5 <i>b</i>	0.4 M-NaCl	97	5
5 <i>c</i>	0.1 M-NaCl	97	11
5 <i>d</i>	0.05 M-NaCl	96	25
5 <i>e</i>	0.005 M-NaCl	96	23
5 <i>f</i>	'Saline tris buffer'	98	15
5 <i>g</i>	'Saline phosphate buffer'	99	36

An illusion of high viability could have occurred if freezing had induced lysis of the suspensions before the organisms were spread on the agar films for slide culture. No signs of lysis were observed on the slide cultures, and if such lysis had been significant it should have been detectable as an optical density change on thawing a frozen suspension. This was not observed: a suspension equiv. 2.2 mg. dry wt. organism/ml. 10 % glycerol had an optical density reading (540 m μ) of 0.258 on dilution (1 vol. 10) in 0.85 % NaCl; a portion of the same suspension frozen in liquid nitrogen and thawed before dilution had an optical density of 0.262.

Effect of freezing on physiology

Other work in our laboratory has shown that populations of logarithmic phase *Aerobacter aerogenes* grown as described here have an initially linear death rate at their growth temperature and pH value in a non-nutrient buffer (see Postgate *et al.* 1961; Fig. 1). Glycerol and other substrates which the organisms metabolize affect the death rate, but diethylene glycol, which is not utilized by this strain, had no effect at all up to 0.2%. The innocuous nature of diethylene glycol enables one to test the effect of deep freezing upon subsequent death. A suspension equiv. 1 mg.

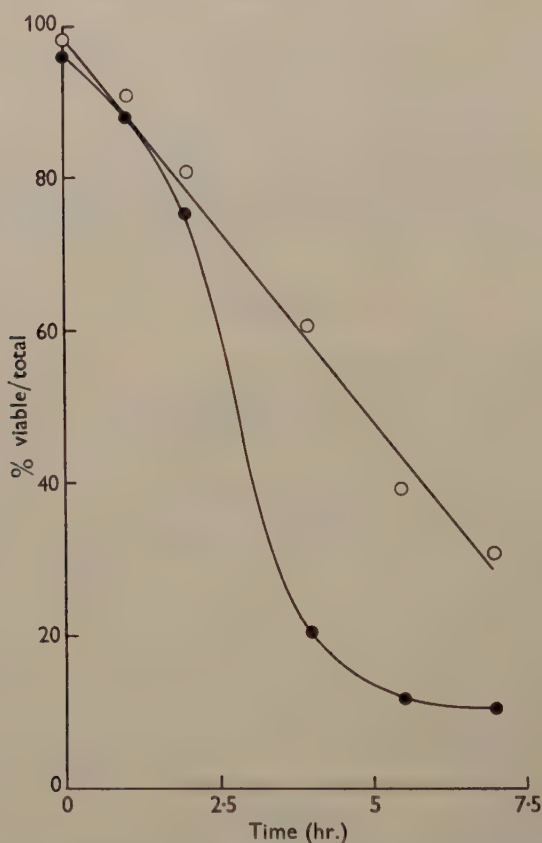


Fig. 1. Death rate of frozen and thawed *Aerobacter aerogenes*. *A. aerogenes* (equiv. 1 mg. dry wt. organism/ml.) grown as in Table 1 were washed by centrifugation, frozen in 10% diethylene glycol at -200° , thawed, diluted 50-fold and aerated in saline 'tris' buffer (pH 7.00 ± 0.05) at 40° (the growth temperature and pH value). Viabilities were determined by slide culture; the control was a similar suspension not frozen. ○, Control; ●, frozen and thawed cells.

dry wt./ml. 10% diethylene glycol was divided in two and one portion frozen in liquid nitrogen and thawed. One ml. of each was diluted into 50 ml. non-nutrient buffer (diethylene glycol finally 0.2%) and incubated with aeration at 40° . Figure 1 shows the viability of samples taken at intervals: the population died more rapidly after deep freezing in the glycol, though the initial viabilities were similar.

Deep freezing might affect the respiratory system. A population (equiv. 10 mg. dry wt./ml.) frozen in 10 % glycerol was thawed in 10 vol. saline phosphate and the Q_{O_2} determined manometrically at 40°; it was $-183 \mu\text{l. O}_2/\text{mg.}/\text{hr.}$ A control treated similarly but stored at -4° in glycerol during the freezing and thawing had a Q value of $-229 \mu\text{l. O}_2/\text{mg.}/\text{hr.}$ In a second experiment the respective values were -243 and $-325 \mu\text{l. O}_2/\text{mg.}/\text{hr.}$

Effect of rate of freezing on survival

Mazur *et al.* (1957) reported that maximum survival of *Pasteurella tularensis* was obtained with slow freezing and rapid warming. In our studies warming was always as rapid as practicable. Some crude experiments were undertaken to seek any effect of freezing rate: 0.2 ml. of a suspension (equivalent to 1 mg. dry wt./ml.) in 10 % glycerol was frozen in a deep freezer at -20° (taking 3 ± 1 min. to solidify) beside 2.3 ml. of a similar suspension (which took about 20 min. to solidify). The specimens were deliberately placed where freezing would be slow. No differences among the viabilities were observed: control, 98 %; quickly frozen suspension, 98 %; slowly frozen, 97 %. Freezing in liquid nitrogen by the procedure we routinely used took 1–2 sec.; freezing in isopentane chilled to -190° took much less than 1 sec. and, though exposure to isopentane killed a few bacteria (97 % viability reduced to 95 %), deep freezing had no subsequent effect (95 % viability).

Penetration by protective agents

The substances tested as protective agents against freezing damage were chosen as three likely to penetrate freely into the cell cytoplasm (glycerol, diethylene glycol, erythritol) and three unlikely to do so on a large scale (glucose, sucrose, polyethylene glycol). It was necessary to know whether in fact these materials behaved as expected. Mager, Kuczynski, Schatzberg & Avidor (1956) showed that the optical densities of live bacterial suspensions in osmotically active solutions were greater than in distilled water, and interpreted this 'optical effect' as an increase in the refractive index of the cytoplasm consequent on its adjustment to the osmotic pressure of the environment. Materials that penetrate to the cell cytoplasm should be osmotically neutral and should show no such effect. The permeability of the bacteria used in this work was examined by using this principle, after preliminary tests had shown that a maximum optical effect occurred with 0.4M-NaCl (optical density in 0.4M-NaCl = 130 % that in distilled H_2O). The optical densities of suspensions equivalent to 0.13 mg. dry wt., 95–100 % viable bacteria/ml. medium were compared in 0.4M solutions and in distilled water; the extents of the optical effect were: glycerol, -4% ; diethylene glycol, -1.6% ; *i*-erythritol, $+2\%$; glucose, $+15\%$; NaCl control, $+34\%$. Some of the optical effect of glucose was doubtless masked by the relatively high molar refractive increment of glucose; with sucrose this property rendered the method inapplicable (optical effect -15%), and its penetration was investigated by using the thick suspension technique of Mitchell & Moyle (1956) and estimating sucrose refractometrically. The sucrose-penetrable space in bacteria centrifuged to constant volume in 0.1M-sucrose was 19 % of their total volume. This value is incompatible with penetration into the cells. Theoretically, the intercellular water between close-packed bacteria should occupy about 26 % of the volume and

the inter + intracellular water about 70 % (Mitchell & Moyle, 1956). Penetration by polyethylene glycol was not studied; it was assumed not to penetrate the organisms because of its high mean molecular weight of 10,000.

Storage of frozen populations

Batches of frozen beads of bacterial suspension were transferred to cold, closed test tubes and stored at -20° in a deep freezer to examine their storage lives with various protective agents. The storage lives were dramatically different (Table 2).

Table 2. *Cold storage of frozen suspensions of Aerobacter aerogenes*

Suspensions of *Aerobacter aerogenes* (equiv. 1 mg. dry wt. organism/ml.) grown as in Table 1 were suspended in the 10 % aqueous solutions below, frozen as beads of about 0.05 ml. in liquid nitrogen and stored in closed tubes at -20° . Individual beads were thawed by dropping into 2.5 ml. buffered saline at 18 to 20° and the viabilities of the thawed suspensions measured by slide culture.

Solute		Storage life at -20°							
		0	2	6	10	20	27	40	
Glycerol	Days								
	Viability (%)	95	92	91	86	92	85	85	
Polyethylene glycol	Days	0	0.7	1.7	4	16			
	Viability (%)	96	80	87	70	65			
Diethylene glycol	Days	0	2	16					
	Viability (%)	98	72	53					
Sucrose	Hours	0	2	5	8	11.7	24		
	Viability (%)	98	78	72	60	25	25		
Glucose	Hours	0	2	4.5	7.5	16.5			
	Viability (%)	92	31	22	19	12			
D-Erythritol	Minutes	0	5	10	15	20	100		
	Viability (%)	94	98	9	3	4	1		
Glycerol*	Days	0	0.7	1.8	4.8	9	15.6	29	61
	Viability (%)	98	94	96	93	94	86	86	78

* In this case the suspension contained equiv. 10 mg. dry wt. organism/ml.

Even after 40 days a majority of those populations frozen in glycerol were viable, whereas after 10 min. the majority of those frozen in erythritol were dead. It is probable that the population taken at 5 min. from the erythritol series had not completely warmed up from the -200° of liquid nitrogen to the -20° of the storage cabinet. Other materials gave intermediate storage lives. The data given in Table 2 are representative of at least two sets of experiments with each storage medium; erythritol was tested three times owing to the exceptionally short storage life observed with it; polyethylene glycol was tested three times since on one occasion death was more rapid than that recorded in Table 2 (viability dropped from 87 to 20 % between the 24th and 40th hours). Beads of glycerol, polyethylene glycol and sucrose took on a damp appearance after 1-3 days' storage; those of erythritol and diethylene glycol did not. None of the beads appeared vitreous after freezing in comparison with a frozen solution of 50 % glycerol. Some indication that the population effect of Major *et al.* (1955) did not occur on storage with glycerol is indicated by the experiment also recorded in Table 2 in which a frozen population equiv. 10 mg. dry wt. organism/ml. survived at -20° in a manner similar to one equiv. 1 mg. dry wt./ml.

DISCUSSION

The mechanism of killing by freezing and of protection of organisms from such injury is complex and has been discussed by several of the workers mentioned in the Introduction. For the particular case of bacteria there is reason to believe that mechanical injury by ice crystals is not responsible (Harrison & Cerroni, 1956). Evidence exists that the major contributory factor is osmotic shock (Harrison, 1956) though Mazur's (1960) findings do not support this view. By analogy with the protective action of glycerol on erythrocytes (Lovelock, 1953), it has been suggested that partial dehydration by penetration of glycerol into the cell cytoplasm is necessary for bacteria to survive freezing (Hollander & Nell, 1954). We shall restrict our discussion to pointing out where certain of our data are relevant to these questions.

The necessity for penetration by the protective substance. With our populations, glycerol, erythritol and diglycol penetrated the bacterial cytoplasm. Glucose and sucrose did not, and it is not likely that the high-molecular weight polyglycol did so. As protective agents all these substances were effective; hence penetration is irrelevant to protection against killing by freezing.

The necessity for partial dehydration. The two classes of protective agent, penetrating and non-penetrating, have in common that they could both dehydrate the cell cytoplasm; one class would do this by osmotic dehydration, the other class would cause a more modest dehydration by physical dilution of the cell cytoplasm. If dehydration were necessary for protection, a high molecular weight substance should have no protective effect owing to its low osmotic pressure in 10 % solution; yet polyethylene glycol protected perfectly well.

Freezing as a form of osmotic damage. The protective action of materials that do not penetrate the organisms yet which were present at initially high molarities (glucose or sucrose) makes it unlikely that concentration of solute during freezing exerted any lethal effect on our populations. In addition, survival in distilled water ought to be maximal if osmotic damage of this kind were relevant, but with our population distilled water was as lethal an environment as broth or the basal medium. This observation conflicts with those recorded by Harrison (1956) and Clement (1961), who found distilled water a relatively innocuous environment for freezing; the difference may be due to our use of a different species, or to the fact that our organisms were in a state corresponding to the logarithmic phase of growth of a batch culture whereas those studied by Harrison and Clement were stationary. The phase of growth is known to influence markedly the sensitivity of bacteria to damage by freezing (Toyokama & Hollander, 1956).

Metabolic damage after freezing and thawing. Though protected populations were still viable after this treatment, it is clear that they sustained some damage since the deep-frozen organisms died more rapidly than controls in starvation conditions and had a reduced rate of substrate oxidation. Squires & Hartsell (1955) reported that freezing and storage altered the lags and growth rates of their bacteria and Straka & Stokes (1959) reported a 'nutritional injury' of frozen bacteria whereby a proportion of the population, after thawing, was found to be nutritionally exacting for materials of a peptide character.

Toxicity of the preserving agent. Though glycerol is widely used as a protective agent, toxic effects at the necessary concentration have been recorded. For example,

Quadling (1960) reported about 39 % survival after exposure of *Xanthomonas phaseoli* to 15 % glycerol broth though the whole 39 % then survived freezing and thawing. Our populations were not affected by 10 or 15 % glycerol (though 30 % aqueous glycerol killed 60 % of the organisms) but in other tests we have observed such toxicity. *Vibrio anguillarum* (NCMB 6) fell in viability from 55 to 40 %; *Chromobacterium violaceum* (NCIB 8182) fell from 92 to 87 %; *Escherichia coli* (Jepp) was uninfluenced (94 % in control, 96 % in 10 % glycerol). With our population of *Aerobacter aerogenes* slight toxicities occurred with certain substitutes for glycerol (Table 1) but, in agreement with Quadling, the survivors of such toxicity were protected from freezing damage.

Distinction between freezing and cold storage. Though several different chemicals protected our organisms against freezing damage, the storage life of a frozen suspension at -20° was very much influenced by the nature of the protective agent; periods of maximum survival ranged from a few minutes in erythritol to many days in glycerol. Speculation about the reasons for these differences would not be fruitful on the data available, but it is obvious by arguments similar to those above that penetration or osmotic dehydration are irrelevant to store life in the frozen state. Our observations suggest that the population effect reported by Major *et al.* (1955) is not involved in the freezing or thawing processes. It is probably a phenomenon that only becomes manifest on storage and even then may not occur in protective environments such as 10 % glycerol.

Effect of freezing rate. In the protective environment of 10 % glycerol we could detect no significant effect of rate of freezing, though our experiments were cruder than those of Mazur *et al.* (1957) in that we knew the freezing rates only roughly. Mazur *et al.*'s experiments were concerned with survival in a non-protective environment, and in such conditions our organism seemed to differ from *Pasteurella tularensis*, since with *Aerobacter aerogenes* slow freezing of 10 ml. samples in buffer in solid CO_2 was more lethal (viability dropped from 95 to 1 %) than rapid freezing of similar samples in liquid N_2 (32–39 % in Table 1).

Record & Taylor (1960) showed that organisms which have been dried in sucrose solutions are subject to internal diffusion pressures on reconstitution due to sugar trapped between cell wall and protoplast membrane. Such diffusion pressures may cause disruption and death of the organisms; high molecular weight substances protect by decreasing the amount of sugar so trapped. During the present work diffusion pressures must have arisen when frozen preparations were in process of thawing, particularly during the experiments involving glucose and sucrose; we attribute the fact that our organisms survived freezing and immediate thawing to our use of solutions which were unlikely to have become as concentrated during this period as they would have done during a treatment that involved drying. The observations reported here help to resolve the paradox mentioned in the introduction. Moses (1955) and Postgate (1960) suspended their organism in very dilute buffer (0.0625 M- KH_2PO_4) and Wade & Lovett (1961) used distilled water containing traces of salts carried over in the washing procedure. These are among the most lethal environments encountered in the present work. Even broth had some protective effect, and the majority of studies in which high survivals have been shown were concerned with organisms suspended in such partially protective environments. Broadly speaking, one might expect maximum destruction of organisms on freezing in

'saline', minimum damage in a 10% solution of a non-electrolyte, intermediate degrees of damage in ordinary bacteriological media, distilled water or tap water.

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A Comparison of Methods for Classifying Rhizosphere Bacteria

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SUMMARY

A comparison was made between the following three methods of classifying bacteria: (1) division on associated characters, (2) identification by the use of Skerman's key, (3) classification by means of Affinity Index. Division on associated characters was of no value in this particular case. Skerman's key was adequate for the identification of the isolates, while the Affinity Index (a slight modification of the Similarity Index of Sneath) gave a comprehensive view of the relationships between isolates. It was considered that a random sample of 43 isolates selected from a collection of 318 rhizosphere bacteria formed a spectrum, rather than a series of groups.

INTRODUCTION

The reviews on rhizosphere bacteria (Clark, 1949; Katznelson, Lochhead & Timonin, 1948; Starkey, 1958) indicate that the successful identification of all the rhizosphere isolates has not yet been achieved. Many workers have devised their own systems of classification, e.g. the nutritional classification (Lochhead & Chase, 1943), morphology and gelatin digestion (Conn, 1948) and morphology, staining and dye tolerance (Clark, 1940). Sperber & Rovira (1959) used Skerman's key (1959) of identification in *Bergey's Manual* (1957) to identify over 80% of their isolates to generic level. The isolates classified as *Arthrobacter* spp. by these authors were divided into 'arthrobacters' and 'arthrobacter-like-nocardias'. In a preliminary comparison between these isolates and type *Arthrobacter* spp. it became evident that there were certain generalized differences between *Arthrobacter* and the rhizosphere isolates. The main difference was in the coccal form; *A. globiformis* breaks up completely into perfect spheres while the rhizosphere isolates formed coccobacilli, with some rods in most older cultures. *A. globiformis* had certain of its stages Gram-positive, whereas the rhizosphere isolates did not show this Gram-variable characteristic; also, there were differences in the metabolic patterns. As a consequence of these observations it was decided to re-examine the complete data collected by Sperber & Rovira on their 318 isolates.

The principle of division on associated characters suggested by Sneath (1957*a*) was attempted with the 318 isolates. It was not possible to use the Similarity Index system of Sneath, as no electronic computer was available. In addition to this re-examination of existing results, 43 cultures were selected at random from the Sperber-Rovira collection, and together with 21 known cultures were subjected to a

further series of tests. The results from these tests were used to group and identify the cultures by three methods, the details of which appear in this paper together with the re-examination of the original results.

METHODS

Organisms. The original collection of rhizosphere isolates of Sperber & Rovira (1959) was maintained at 4° and subcultured on yeast extract-peptone-soil extract (YPS) agar (Bunt & Rovira, 1955) at intervals of 6 months. The named cultures from various laboratories listed below were:

Achromobacter A1, *Achromobacter* A2 (University of Melbourne); *Agrobacterium radiobacter* NCIB 8149 (Department of Scientific and Industrial Research, England); *Arthrobacter aureescens*, *A. citreus*, *A. globiformis*, *A. simplex*, *A. ureafaciens* (Department of Agricultural Science Service Laboratories, Ottawa, Canada); *Bacillus subtilis* NCTC 6342, *Flavobacterium* sp. (University of Melbourne); *Micromonospora* sp. (University of Sydney); *Nocardia asteroides* NCTC 6761, *N. rubra* (University of Sydney); *Pseudomonas fluorescens* (University of Adelaide); *P. medicaginis*, *P. syringae* (University of Melbourne); *Rhizobium trifolii* (from D. Norris, C.B. 721 from *Trifolium polymorphum*), *R. meliloti* (Q.A. 867 from Rothamsted), *Rhizobium* sp. (from G. D. Bowen Q.A. 549 from *Centrosema pubescens*); *Streptomyces venezualae* (University of Sydney); *Xanthomonas campestris* (University of Melbourne).

Basal medium. The yeast-extract peptone nitrate broth of Sperber & Rovira (1959), with 15 g. agar/l. for solid medium, was used.

Gelatin agar. Gelatin (0.4%, w/v) in basal agar. Hydrolysis was observed by flooding the plates with saturated ammonium sulphate following incubation at 26° for 7 days.

Starch agar. Starch (0.2%, w/v) in basal agar. Hydrolysis was observed by flooding the plates with iodine solution after 7 days at 26°.

Casein agar. Ten ml. of sterile 9% (w/v) solution of powdered milk were added to every 100 ml. basal agar immediately before pouring plates. Hydrolysis was observed as a clearing round the colony.

High pH medium. Difco veal infusion (23 g.) and 15 g. agar/l. water; finally adjusted to pH 8.5.

Nutrient agar. Oxoid nutrient agar.

Apatite agar. As used by Sperber (1958).

Peptone yeast extract (PY) broth. Peptone (10 g.), Difco yeast extract (5 g.), sodium chloride (5 g.)/l. water. H₂S production was determined with lead acetate filter paper strip in the mouth of the tube.

Methylene blue agar. Basal agar + 1% (w/v) glucose and 1 ml./l. of 0.5% (w/v) methylene blue was tubed in 10 ml. lots and inoculated before setting. Those isolates which reduced more than half the depth of the agar were considered positive.

Sucrose mannitol sauerkraut (SMS) broth. Sucrose (5 g.), mannitol (5 g.), sauerkraut juice (20 ml.), sodium citrate (1 g.), magnesium glycerophosphate (1 g.), Casamino acids (1 g.), ammonium nitrate (0.1 g.), distilled water 1000 ml. (Harris, J. R., personal communication). This medium was used to demonstrate fluorescence when the selected cultures were retested.

Ulrich's milk. The reaction was recorded after 15 days at 26° (Ulrich, 1944).

Glucose broth. Ten g. glucose, 1 g. Difco yeast extract, 0.4 g. K_2HPO_4 , 0.5 g. KNO_3 , 0.05 g. $MgSO_4 \cdot 7H_2O$, 0.1 g. NaCl, 0.17 ml. 6% (w/v) solution of ferric citrate, 4 ml. indicator (equal parts of saturated aqueous bromocresol purple and cresol red) in 1 l. water.

Sucrose broth. As for glucose broth but with 10 g. sucrose. One series of sugar broths was incubated aerobically, the other anaerobically by the steel wool technique of Parker (1955).

Sensitivity to antibiotics. This was tested by surface seeding basal agar plates with cultures and then placing one Oxoid 'Multodisk' (11-14D)/plate. Each disk contains eight peripheral disks—one for each of the following: chloramphenicol (50 $\mu g.$), erythromycin (50 $\mu g.$), sulphafurazole (500 $\mu g.$), novobiocin (30 $\mu g.$), oleandomycin (10 $\mu g.$), penicillin (5 units), streptomycin (25 $\mu g.$), tetracycline (50 $\mu g.$). The zones of inhibition were recorded after incubation for 7 days at 26°.

Examination of original data

The data previously collected by Sperber & Rovira (1959) from a series of 19 tests on 318 rhizosphere isolates were used. The two tests, namely, colour and fluorescence which were performed on two media were recorded as follows: colour 1 was presence of pigment in colonies on apatite agar; colour 2 was presence of pigment on nutrient agar; fluorescence 1 was obtained on yeast extract mannitol agar; fluorescence 2 on nutrient agar.

The results were transferred to edge-punched cards, one card for each isolate. Holes were punched for positive values and left blank for negative values. The total number of isolates positive for each test was counted, and those tests which did not have some positive and some negative isolates were discarded. Three tests were discarded for this reason, namely, the breakdown of cellulose (all negative), the nodulation of subterranean clover (all negative) and growth in peptone water (all positive).

A 2×2 table was set up for each combination of the remaining 16 tests and χ^2 values calculated for each of the 120 tables. When the association was significant at the 1% level, the degree of association was measured by using Yule's coefficient of association (the Q value). Moroney (1957) set out the methods used in these calculations. Sneath (1957a) has pointed out that the associations revealed by these calculations may be due to two expressions of the one character, and when discovered such associations should be removed. For this reason the closely associated characters 'dissolving of apatite' \times 'acid production' (Sperber, 1958) and 'colour 1' \times 'colour 2' were removed from the calculations.

RESULTS

Examination of original data

The data obtained by Sperber & Rovira (1959) from 16 tests on 318 rhizosphere isolates were examined by the methods set out above. Table 1 shows the Q values for these isolates and the algebraic sum of the Q values for each test. The highest Q value in Table 1, -0.86, was for Gram reaction against growth on high pH medium. Of the 249 isolates which grew on the high pH medium only 19 were Gram-positive. All the other Q values were low, the lowest being only -0.29. The total Q values

(disregarding sign) for ammonia production and colour 1 were similar. Colour 1 was significantly associated with eight other characters (disregarding colour 2), whereas ammonia production was associated with seven other characters. Colour 2 had a much lower total Q value than colour 1. The selection of a character for division was difficult; those characters strongly associated with some characters, namely, Gram-reaction and colour 2, were not associated with enough characters to give them a high total Q value. The characters which were associated with a large number of other characters had high total Q values, even though they had only moderate or low degrees of association.

Table 1. *Q values significant at the 1% level for 318 rhizosphere isolates*

Only half the table of Q values is shown, the blank part being the mirror image of the part below. The total number of isolates positive for each test and the algebraic sum of the Q values are also shown. C1 = colour 1, Ap = solution of apatite, Ac = acid production from glucose, Gr = Gram reaction, $H_2S = H_2S$ production from peptone, $NH_3 = NH_3$ production from peptone, $NO_2^- = NO_2^-$ production from nitrate, Mo = motility, Ca = catalase, Ge = gelatine hydrolysis, F1 = fluorescence 1, pH = growth on high pH medium, C2 = colour 2, F2 = fluorescence 2, Y = presence of Y forms. Pl = presence of pleomorphic forms.

	Total positives	C1	Ap	Ac	Gr	H_2S	NH_3	NO_2^-	Mo	Ca	Ge	F1	pH	C2	F2	Y
C1	112															
Ap	126	0.37														
Ac	204	—	—													
Gr	55	-0.32	—	—												
H_2S	117	0.48	—	-0.44	—											
NH_3	238	0.50	0.45	—	—	0.49										
NO_2^-	83	0.33	—	—	—	0.37	0.56									
Mo	197	-0.29	—	-0.39	0.63	—	—	—								
Ca	221	0.50	0.50	—	—	—	0.35	—	—							
Ge	147	—	0.59	0.56	—	—	0.49	—	—	—						
F1	79	-0.49	-0.40	—	—	—	—	—	—	-0.48	-0.39					
pH	249	—	—	—	-0.86	—	—	—	0.51	—	—	—				
C2	206	—	—	—	—	—	0.45	—	-0.40	0.64	-0.63	—	—			
F2	31	—	—	—	—	—	—	—	—	—	—	—	-0.69	—		
Y	174	—	0.39	—	-0.38	—	—	—	—	—	—	—	—	—	—	
Pl	77	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.53
Total Q values		3.28	2.70	1.39	2.19	1.78	3.29	1.26	2.22	2.47	2.66	1.76	2.06	2.12	0.69	1.30

The most important character should have a large number of other characters strongly associated with it, therefore the character which had the highest algebraic sum of its Q values was considered the most important for division. Table 1 shows that ammonia production had the highest total Q value (3.29), so the isolates were divided on ammonia production (see Fig. 1). The two resulting groups, the ammonia-producing group and the non-ammonia-producing group, were treated in exactly the same manner as the original isolates, i.e. all possible associations were tested for significance, and those found significant at the 1% level measured by the Q test. The resultant Q values were then algebraically summed and the character with the highest algebraic sum of Q values used for the next division. This character was gelatin hydrolysis for the ammonia-producing group, and growth on high pH media for the non-ammonia-producing group. Division was continued in this way until there was no character that was significantly correlated with any other two characters.

Table 2. *Q values significant at the 5% level for 64 bacteria*

Total positive		pH	Ge	Ca	St	Ap	Me	Po	UA	UB	NH ₃	NO ₂ ⁻	H ₂ S	Ga	Gn	Sa	Sn	F	C1	C2	Ch	Er	Su	No	Ol	Pe	St	Gr	Fi	Y	Pl	Co
pH	54																															
Ge	33	—																														
Ca	19	—	0.95*																													
St	23	—	—	0.77																												
Ap	21	—	—	—	—																											
Me	26	—	—	0.58	—	—																										
Po	31	—	—	0.53	0.49	—	—																									
UA	13	—	0.61	—	—	—	—	—																								
UB	32	—	-0.52	-0.46	—	—	—	—	—																							
NH ₃	57	0.84*	—	—	—	—	—	—	—	—																						
NO ₂ ⁻	21	—	-0.62	—	—	—	—	—	—	—	—																					
H ₂ S	23	—	—	—	—	—	—	—	—	0.56	—	—																				
Ga	28	—	—	0.51	—	—	—	—	0.93*	-0.74*	—	—	—																			
Gn	9	—	—	—	—	—	—	—	—	—	—	—	—	0.99*																		
Sa	23	—	—	—	—	—	—	—	0.81*	-0.62*	—	—	—	0.87*	0.98																	
Sn	9	—	—	—	—	—	—	—	—	-0.82	—	—	—	—	—	—																
F	14	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—															
C1	40	—	—	0.58	—	—	—	—	—	—	—	—	—	—	—	—	—	—														
C2	26	—	—	—	—	—	—	—	—	-0.50	—	—	—	—	—	—	—	—	—													
Ch	54	—	—	—	—	-0.85*	—	—	—	—	—	—	—	—	-0.75	—	-0.74	-0.79	—	—												
Er	41	—	—	—	0.61	—	—	—	—	—	—	—	—	0.53	—	—	—	—	—	—	1.00*											
Su	41	—	—	—	0.73*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.83	0.89*										
No	45	—	—	—	—	-0.75	—	—	—	—	—	—	—	—	—	—	—	-0.65	—	—	1.00*	0.84*	—									
Ol	14	—	—	—	0.65	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.00*	0.82	—								
Pe	14	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.72							
St	56	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.89*	0.82	—								
Gr	11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.00	—	—	0.84*	0.84	—					
Fi	19	—	—	0.52	0.58	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Y	17	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Pl	11	—	—	—	—	—	—	-0.69	—	—	-0.91*	—	—	—	—	—	—	—	-0.80	-0.72	—	—	1.00	—	—	—	—	—	—	-1.00	0.77*	—
Co	21	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.70	—	—	—	—	—	—	—
Total Q values		0.84	2.70	4.90	3.83	1.60	0.58	1.71	2.35	4.22	1.75	0.62	0.56	4.57	2.72	3.28	1.56	1.44	1.38	1.22	5.96	6.76	5.09	3.24	4.73	1.56	1.71	2.68	2.10	0.77	5.89	0.70

Those Q values marked * were significant at 1 %. Only half the table is shown, the part left blank being the mirror image of the part below. The total number of bacteria positive for each test and the algebraic sum of the Q values are shown.

pH = growth in high pH medium, Ge = gelatin liquefaction, Ca = casein digestion, St = starch hydrolysis, Ap = apatite solution, Me = methylene blue reduction, Po = growth on potato, UA = acid production in Ulrich milk, UB = alkali production in Ulrich milk, NH₃ = NH₃ production from peptone, NO₂⁻ = NO₂⁻ pro-

duction from NO₃⁻, H₂S = H₂S from peptone, Ga = aerobic; acid from glucose, Gn = anaerobic; acid from glucose, Sa = aerobic; acid from sucrose, Sn = anaerobic; acid from sucrose, F = fluorescence (SMS), C1 = colour 1, C2 = colour 2, Ch = chloramphenicol sensitivity, Er = erythromycin sensitivity, Su = sulphafurazole sensitivity, No = novobiocin sensitivity, Ol = oleandomycin sensitivity, Pe = penicillin sensitivity, St = streptomycin sensitivity, Gr = Gram reaction, Fi = filamentous forms present, Y = Y forms present, Pl = pleomorphic forms present, Co = coccal forms present.

The Q value table for the first division (Table 1) is the only one presented here in order to conserve space. The six groups which resulted from this division on associated characters are given in Fig. 1, but a comparison with the identification by Sperber & Rovira (1959) showed that each group contained several genera. In view of the heterogeneity of the groups, the second part of this investigation was undertaken using typed cultures as well as the selected rhizosphere isolates.

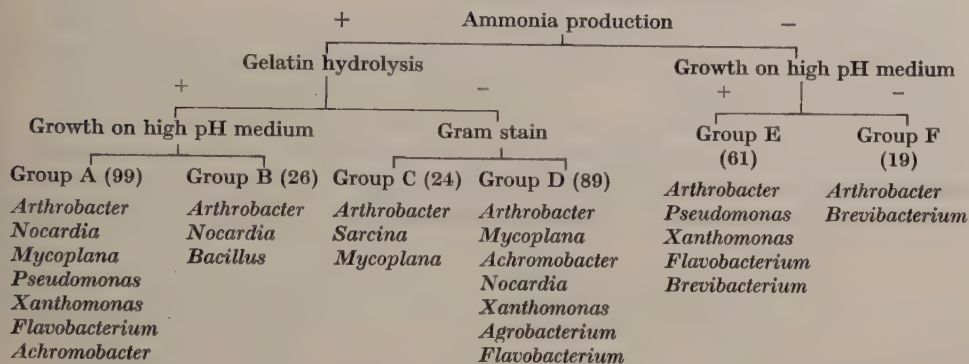


Fig. 1. Division of the 318 rhizosphere isolates using associated characters as shown. Positive reactions are on the left, negative reactions on the right. The number of isolates in each group is shown in brackets and the principal genera as identified by Sperber & Rovira (1959) are listed for each group.

Retesting of selected bacteria

The morphological, cultural, biochemical and antibiotic sensitivity characteristics of 21 named cultures and 43 rhizosphere isolates were studied. Forty-one characters were recorded as positive or negative on edge-punched cards. Of these characters, thirty-one were used to determine the Q values (Table 2). The most notable feature of Table 2 was the small number, 28, of associations significant at the 1% level, as compared with 35 in Table 1, even though the number of tests had risen from 16 to 31 increasing the number of possible associations from 120 to 535. The decrease in numbers of significant associations at the 1% level can be attributed to the lower number of organisms used in the second case, making it less likely to obtain significant results. For this reason the significance level was set at 5% and the Q values of Table 2 have been worked out for associations significant at this level.

A decision had to be made as to which of the associations should be removed in accordance with the principles laid down by Sneath; to conform with Table 1 the associations of colour 1 × colour 2 and dissolving of apatite × acid production were removed. The removal of other associations may be justified, but, as it was considered that inclusiveness was better than exclusiveness no further associations were removed. In comparing Table 1 with Table 2, the only common associations found were colour 1 × colour 2; dissolving of apatite × acid production, and pleomorphism × Y forms. The association of growth at high pH value × Gram reaction, which was the highest in Table 1, does not appear in Table 2, indicating that the association probably was spurious. When this association was disregarded the Q values (ignoring sign) of Table 1 ranged from 0.29 to 0.69 and those in Table 2 from 0.46 to 1.00.

If the levels of significance in both tables had been adjusted so that only Q values above a given figure (say 0.8) were recorded, then there would be more associations in Table 2. The greater range of bacteria and tests used for Table 2 accounted for the greater number of high associations in Table 2 than in Table 1, e.g. the association between Gram-positivity and erythromycin susceptibility could be demonstrated only when there was sufficient Gram-positive organisms and when the test for erythromycin susceptibility was performed.

A system of division was built up for the 64 bacteria by using the methods described for Fig. 1, the only difference being that a 5% level of significance was used. These results appear in Fig. 2. Erythromycin susceptibility had the highest total Q value (6.76) in Table 2, and was used to make the first division in Fig. 2. This is a good character to divide on, as it has a large number of characters strongly associated with it. Pleomorphic forms also had a high total Q value of 5.89. Five groups resulted from the system of division set out in Fig. 2. The fact that three out

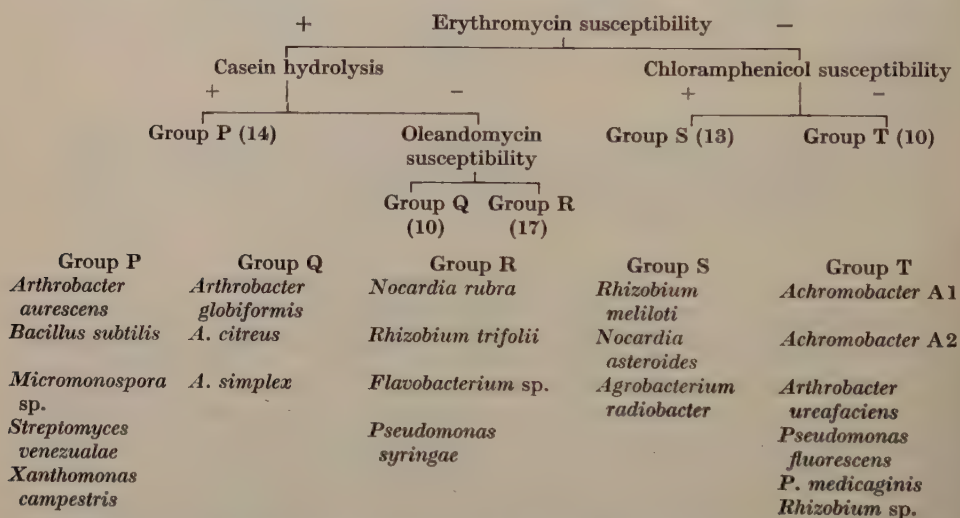


Fig. 2. Division of 64 bacteria on the basis of associated characters. Positive reactions are on the left, negative reactions on the right. The number of bacteria in each group is shown in brackets. The named bacteria within each group are also shown.

of the four characters used for division were reactions to antibiotics was not entirely due to associations between the various antibiotics. In the group of 23 bacteria that formed the erythromycin-resistant group, chloramphenicol-sensitivity was negatively associated with: dissolving of apatite, acid production in Ulrich's milk, aerobic production of acid from glucose, anaerobic production of acid from sucrose, and fluorescence; and it was positively associated with novobiocin sensitivity. In the group of 27 bacteria sensitive to erythromycin and not attacking casein, oleandomycin sensitivity was positively associated with the breakdown of starch, the solution of apatite and the presence of cocci.

The groups formed in Fig. 2 have been labelled P, Q, R, S, T, to facilitate comparison with the groups obtained from the 318 isolates (Fig. 1). The named cultures have been listed under the groups to which they belonged. In general each group

contained several genera except for group Q, in which only three *Arthrobacter* spp. occurred.

A comparison of the groupings of the 43 rhizosphere isolates common to Fig. 1 and Fig. 2 is given in Table 3. This shows a poor coincidence of groups, e.g. group Q isolates identified as *Arthrobacter* spp. included members from groups A, D, E, and F. Therefore it was impossible to use Fig. 2 to identify any of the groups A to F from Fig. 1.

Owing to the failure of the system of division on associated characters to give satisfactory groupings of the named cultures, the 64 cultures were traced through

Table 3. *A comparison of the groupings of the 43 isolates common to both Fig. 1 and Fig. 2*

Groups from Fig. 2	Groups from Fig. 1						Totals
	A	B	C	D	E	F	
P	6	1	—	1	1	—	9
Q	1	—	—	3	1	2	7
R	3	2	—	4	4	—	13
S	1	—	1	5	3	—	10
T	2	—	—	2	—	—	4
Totals	13	3	1	15	9	2	43

Skerman's key of *Bergey's Manual* (1957); the results are given in Table 4. Of the seven rhizosphere isolates which were considered to be *Arthrobacter* spp. in Fig. 2, only one was identified as such by the key. It would appear that many of the named isolates were incorrectly identified. This was the fault of the testing procedures rather than of the key, e.g. *Rhizobium* spp. fell into the *Pseudomonas-Alcaligenes* group because there was no satisfactory way of identifying *Rhizobium* except on its ability to nodulate legumes, a test which is virtually impossible to perform against all legumes. The lack of a successful routine technique for determining flagellation was also a great handicap.

Although the use of Skerman's key resulted in the naming of organisms, quite different isolates often appeared to belong to the same genus because they were identical only in the characters listed in the key, while they varied in a great number of other characters. This arbitrary nature of the key exists because of the lack of comparative studies with a wide range of organisms. Sneath's method of calculating Similarity Index would seem the ideal way of making such comparative studies. It was therefore decided to modify the method slightly so that it could be used for these isolates without the use of an electronic computer. The modifications made were that negative similarities were considered as important as positive similarities, and all characters which were entirely positive or entirely negative were omitted. Of the forty-one characters recorded there were thirty-nine that could be used on this basis. To avoid confusion the modified index was called the 'Affinity Index' (AI) and it may be expressed as

$$\text{Affinity Index} = \frac{\text{No. of characters in common}}{\text{Total No. of characters considered}} \times 100 \%$$

The punch cards which had been prepared for determination of associated characters in the 64 isolate groups were used to determine the Affinity Indices. The denominator

Table 4. *The identification of 64 bacteria according to Skerman's key*

<i>Achromobacter</i>	<i>Arthrobacter</i>	<i>Bacillus</i>	<i>Brevibacterium</i>	<i>Flavobacterium</i>	<i>Nocardia</i>	<i>Micromonospora</i>	
SR* 60 (ER)	SR 16 (ER) SR 29 (AQ) SR 60 (AT) SR 195 (ER) SR 311 (DT)	<i>B. subtilis</i> SR 15 (FQ) SR 80 (NR) SR 221 (FQ)	SR 12 <i>b</i> (BR) SR 78 (AP) SR 82 (AP) SR 89 <i>b</i> (AP) SR 108 <i>a</i> (DS) SR 198 (DQ) SR 231 (DP) SR 242 (DQ) SR 266 (AP) SR 270 (AQ) SR 281 (DR) SR 315 (DQ)	<i>N. asteroides</i> <i>N. rubra</i>	<i>Micromonospora</i> sp.		
	<i>A. aureus</i> <i>A. simplex</i> <i>A. globiformis</i> <i>A. citreus</i>						
<i>Pseudomonas</i> or <i>Achromobacter</i>	<i>Pseudomonas</i> or <i>Alcaligenes</i>	<i>Pseudomonas</i> , etc. Page 1020, no. 58	<i>Sarcina</i>	<i>Streptomyces</i>	<i>Xanthomonas</i> or <i>Alcaligenes</i>	<i>Xanthomonas</i> or <i>Flavobacterium</i>	Not grouped
SR 3 (DR) SR 83 (DT) SR 143 <i>b</i> (ES) SR 166 (ER) SR 225 (CS)	SR 139 (EP) SR 156 (DR) SR 227 (DS) SR 232 (AT) SR 234 (ES)	SR 294 (AR) <i>Achromobacter</i> A1 <i>Achromobacter</i> A2 <i>Arthrobacter ureafaciens</i> <i>P. fluorescens</i>	SR 12 <i>a</i> (BP)	<i>S. venezuelae</i> SR 63 <i>b</i> (DS) SR 127 (AR) SR 202 (DS) SR 215 (DR) SR 236 (DS) SR 245 <i>b</i> (AS) SR 267 <i>b</i> (ES)	SR 75 (AP) SR 194 <i>b</i> (AR) <i>Flavobacterium</i> sp.	SR 46 (AP)	
<i>Ps. medicaginis</i> <i>Ps. syringae</i>	<i>Agrobacterium radiobacter</i> <i>Rhizobium trifolii</i>				<i>X. campestris</i>		

* Cultures selected from Sperber-Rovira collection are prefixed SR.

Column headings indicate the genus or group of genera to which the bacteria belong. The groupings from divisions in Fig. 1 and Fig. 2 respectively follow the rhizosphere isolate number.

of the expression given above was constant at 39, i.e. the number of tests having some positive and some negative values, and the numerator was determined for each possible pair of isolates. The border of the first card was stained red and compared with the second and subsequent unstained cards. Comparison was made by placing the red card 1 on white card 2 when any test positive (punched) on 1 but negative (not punched) on 2 showed as a white space in a red gap and these spaces were counted. The two cards were then turned over so that white card 2 now lay on red card 1 and any test positive on 2 but negative on 1 showed as a red space filling a white gap. These spaces were counted. The sum of these two totals, which gave the number of tests different between 1 and 2, was recorded. The number of tests in common was the total number (39) less the number of tests which differed. Having obtained the number of tests which differed between cards 1 and 2, card 1 was then compared with card 3 and so on until 1 had been compared with all the cards. Card 2 was then stained and compared with all the unstained cards. In this way over 2000 Affinity Indices were calculated, a difference of four or less characters corresponded to an Affinity Index of 90 %, a difference of five or six characters corresponded to an Affinity Index of 85 % and a difference of seven or eight characters corresponded to an Affinity Index of 80 %. Having obtained Affinity Indices in this way, the results were sorted in the manner suggested by Sneath, the most closely related being put together first and the grouping extended as the relationship decreased. The relationships discovered are shown in a linear manner in Table 5. The name or SR number of the isolate is given in the left-hand column, the code number used in this investigation is shown in the second column, then follows a list of the code numbers of all cultures with an Affinity Index of 80 % or more. Thus *Rhizobium* sp. isolated from *Centrosema pubescens* Benth. was related with an 80 % Affinity Index to SR 225 and to *Rhizobium meliloti*. The cultures at the extreme ends of Table 5 were unrelated to each other or to the cultures in the centre of the table and their order was of no taxonomic significance. The normal way of showing relationships of this type, as used in other papers (Sneath, 1957*b*; Sneath & Cowan, 1958; Hill, 1959) is set out in Fig. 3, in which the squares show those cultures which differ by no more than eight characters and the triangles show those cultures which are dissimilar by twenty or more characters.

A sorting which resulted in a good arrangement was typified by having related isolates close together. When this occurred in Fig. 3 the dark squares representing high affinity lay close to the diagonal. Although this condition was reasonably fulfilled, there was no block of closely related organisms, which indicated that there were no well defined groups of isolates. There was a large group of isolates in the centre, composed mainly of rhizosphere isolates which formed a spectrum of related bacteria rather than a series of related groups. The known bacteria which had the highest affinity to this large spectrum were *Agrobacterium radiobacter* (no. 51), *Xanthomonas campestris* (no. 70), and two *Rhizobium* sp. (nos. 65 and 66). Above the large group was a small group consisting of *Achromobacter* A1 and A2 (nos. 49 and 50), *Flavobacterium* sp. (no. 58), *Pseudomonas fluorescens* (no. 62), *Arthrobacter ureafaciens* (no. 56) and SR 83 (no. 15). The culture which was labelled '*Arthrobacter ureafaciens*' differed in morphology, colony colour, motility, hydrogen sulphide and acid production from the description for this organism given by *Bergey's Manual* (1957). With the exception of *Flavobacterium* sp. which was poorly chromogenic

Table 5. *The relationships between 64 cultures according to the Affinity Index*

Column 1 shows the name or number of the culture and column 2 the code number. On the right of the table are shown the code numbers of all the bacteria having 80 % or more affinity with each of the 64 cultures. Italic figures indicate 85 % affinity, bold figures 90 % affinity.

Name or no.	Code no.	
SR 60	8	
<i>Arthrobacter aurescens</i>	52	
<i>Rhizobium</i> sp.	68	32, 66
SR 294	45	23, 44
SR 311	47	51
<i>Achromobacter</i> A1	49	50
<i>Achromobacter</i> A2	50	49, 56, 62
<i>Flavobacterium</i> sp.	58	62, 56
<i>Pseudomonas fluorescens</i>	62	56, 15, 58, 50
<i>Arthrobacter ureafaciens</i>	56	62, 15, 50, 58
SR 83	15	56, 62, 63
SR 245 B	40	33
<i>Pseudomonas syringae</i>	64	33
SR 225	32	3, 20, 66, 68
<i>Arthrobacter citreus</i>	53	13
<i>A. globiformis</i>	54	55
<i>A. simplex</i>	55	54, 23
<i>Pseudomonas medicaginis</i>	63	10, 15
SR 69	10	23, 65, 13, 22, 44, 63
SR 166	23	10, 65, 22, 1, 26, 55, 45, 66
<i>Rhizobium trifolii</i>	65	23, 10, 13, 22, 66, 1, 5, 19, 36
<i>R. meliloti</i>	66	5, 36, 37, 65, 23, 32, 68
SR 80	13	5, 53, 65, 10, 54
<i>Agrobacterium radiobacter</i>	51	22, 30, 42, 17, 33, 5, 20, 37, 47
SR 156	22	30, 51, 23, 33, 1, 42, 65, 10, 21, 70
SR 215	30	22, 33, 51, 70, 1, 5, 9, 42, 43, 46
SR 227	33	20, 30, 21, 22, 1, 36, 40, 42, 43, 51, 64
SR 134 B	20	33, 3, 43, 1, 5, 19, 9, 32, 36, 42, 51
SR 301	46	44, 43, 14, 16, 34, 70, 1, 3, 11, 12, 19, 30
SR 281	44	43, 46, 1, 3, 19, 20, 46, 33, 48, 5, 9, 26, 30
SR 270	43	44, 1, 3, 19, 20, 46, 33, 48, 5, 9, 26, 30
SR 127	19	3, 43, 5, 9, 20, 26, 46, 65
SR 12 B	3	5, 19, 20, 26, 43, 32, 44
SR 195	26	3, 23, 1, 5, 9, 19, 37, 42, 43, 48
SR 3	1	5, 37, 43, 22, 23, 33, 44, 61, 3, 20, 26, 30, 36, 42, 46, 65
SR 16	5	37, 13, 66, 1, 3, 42, 6, 19, 20, 26, 30, 36, 43, 51, 53, 65
SR 236	37	5, 42, 17, 1, 25, 66, 3, 26, 36, 48, 51
SR 267	42	37, 17, 5, 51, 22, 25, 33, 70, 1, 3, 20, 26, 30, 35, 61
SR 108 A	17	37, 42, 70, 35, 51, 21, 25
SR 232	35	21, 17, 70, 42
SR 139	21	35, 33, 70, 17, 22
<i>Xanthomonas campestris</i>	70	16, 17, 21, 30, 35, 42, 46, 14, 22, 34
SR 89 B	16	14, 34, 70, 41, 46
SR 82	14	16, 34, 46, 70
SR 231	34	14, 16, 11, 12, 46, 44, 70
SR 234	36	33, 66, 1, 5, 20, 37, 65
SR 63 B	9	28, 19, 20, 26, 30, 43, 3
SR 194 B	25	37, 42, 17, 69
SR 75	11	12, 34, 46
SR 78	12	11, 34, 46
SR 315	48	39, 43, 26, 27, 37, 44
SR 242	39	4, 48, 44

Table 5 (cont.)

Name or no.	Code no.	
SR 15	4	31, 39, 44, 27
SR 221	31	4, 44
SR 198	27	4, 48
SR 266	41	14, 16, 44, 59
SR 29	6	5
<i>Nocardia rubra</i>	61	1, 42
<i>Streptomyces venezuelae</i>	69	25
SR 202	28	9, 33
<i>Micromonospora</i> sp.	59	41
SR 12A	2	
<i>Bacillus subtilis</i>	57	
<i>Nocardia asteroides</i>	60	

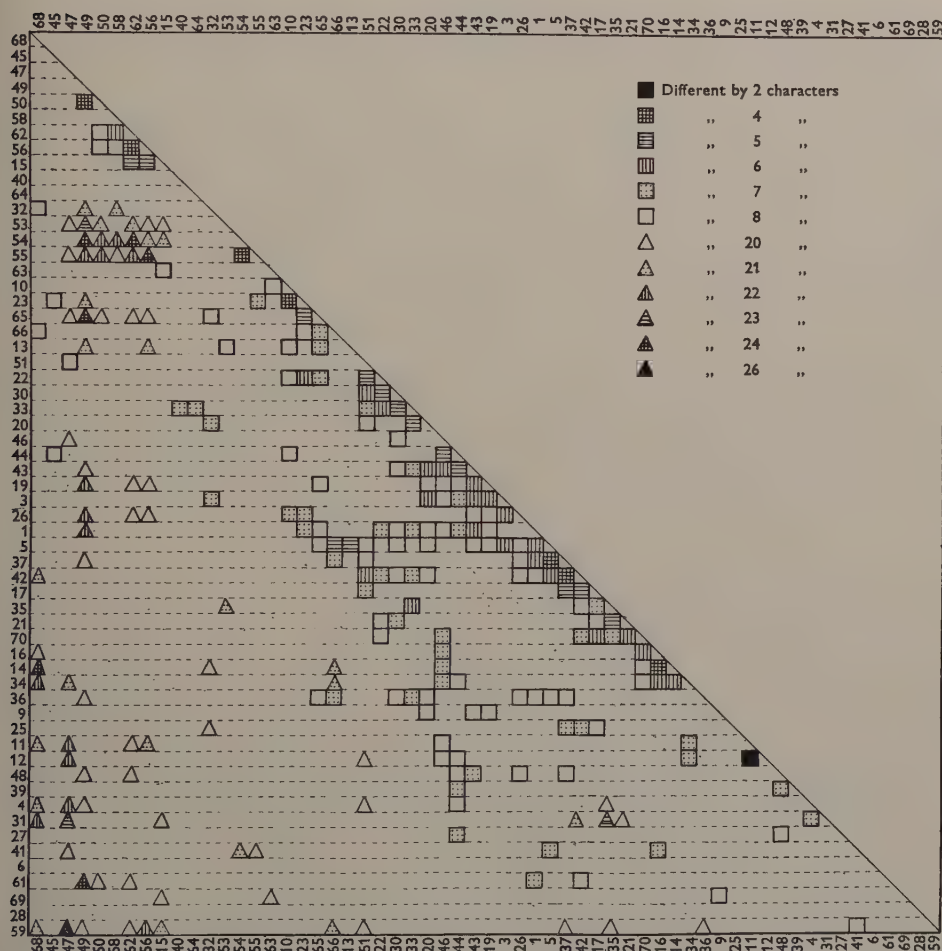


Fig. 3. Diagram showing the relationship between 64 cultures. The shading of squares indicates the number of characters by which similar cultures differ. The triangles indicate the number of characters by which dissimilar cultures differ. Intermediate relationships are not shown. The key for the culture numbers or names is given in Table 5.

and non-proteolytic all other named cultures corresponded closely with the descriptions given by *Bergey's Manual* (1957). Thornley (1960) reported that *Pseudomonas* could be differentiated on the basis of the anaerobic breakdown of arginine. All 64 cultures were tested for anaerobic arginine breakdown and of those which were Gram negative, penicillin resistant, and produced acid from glucose aerobically only six gave a positive arginine test. Those cultures were *Pseudomonas fluorescens*, *Pseudomonas medicaginis*, *Achromobacter* A1 and A2, *Arthrobacter ureafaciens*, and the rhizosphere isolate RS 83. Thus, according to Thornley, the so-called *Arthrobacter ureafaciens*, *Achromobacter* cultures, and SR 83 are really members of the genus *Pseudomonas*. It is interesting to note that in Table 5 these cultures have been grouped together on the basis of Affinity Index quite independently of the arginine test and showed a very poor relationship to the majority of the rhizosphere isolates.

DISCUSSION

The system of division on associated characters proved to be of no value in this particular case, firstly because of the lack of distinct groups within the range of bacteria considered, and secondly because of the somewhat complex nature of the analytical procedure. While it is true that where groups differ in many characters these characters will be associated, it is not necessarily true that where there are associated characters there will be taxonomic groups. It is considered that it is only rarely that the elaborate computation of associations as performed in this paper will be necessary. A simpler process would be to obtain groups by using the Affinity Index and to find differences between groups by inspection.

The best system for identifying the isolates is undoubtedly Skerman's key especially when the type cultures of the genera most likely to be encountered are included with the isolates. The key has several deficiencies. Firstly, it is based on *Bergey's Manual* and any deficiencies in the *Manual* are reflected in the key. Secondly, there is the difficulty in the standardization of testing procedures, which should largely be solved by using *A Guide to the Identification of the Genera of Bacteria* (Skerman, 1959). The present paper arose out of this difficulty of technique standardization. Sperber & Rovira (1959) demonstrated the presence of branching forms in many cultures which would normally be considered to have unbranched forms. This gave rise to their large groups of 'Arthrobacter' and 'Arthrobacter-like-Nocardias', which upon re-examination and comparison with known cultures of these genera proved to be incorrectly identified. Nevertheless, the prevalence of branching amongst the rhizosphere cultures was an important observation and a further study of this phenomenon with type cultures seems necessary.

The relationships between bacteria are well shown by the use of Sneath's Similarity (or Affinity) Index. The limitations of this method lie in the sorting procedures and the selection of tests or characters to be used. A close examination of Fig. 3 shows that several cultures have in fact been missorted, and correction would require a rearrangement of the figure. However the authors believe this is not justified as a perfect arrangement is not possible. As there are only two positions equidistant from any organism in the linear order, a compromise must be used if there are more than two organisms with an equal relationship to any other organism. The presentation of the data as in Table 5 simplifies the sorting procedures as the relation-

ships are more easily seen than in Fig. 3. The other limitations of this method lie in the selection of tests or characters to be used. If the natural groupings of the bacteria considered differ from one another in ten characters, then the chances of determining the groups will depend on how many of the ten good tests are selected and how many poor tests are added to the good tests. The larger the number of poor tests the more the result will tend towards one diffuse group. In discussing test selection for taxonomic purposes, Krassilnikov (1959) pointed out that a good test must be a repeatable test. He suggested that the loss of a character was not as important as the spontaneous gain of a character, and also emphasized the importance of morphology in taxonomy. Cowan (1955) posed the question that bacteria form a spectrum of gradually merging forms. In trying to determine whether this is the case for the rhizosphere bacteria, the two factors to be borne in mind are that the inclusion of too many poor tests would tend to give this result and that the demonstration of a range of bacteria is not sufficient to say that bacteria do not occur as natural taxonomic groups. If any of the types in the range is rare then this would be the place to make a taxonomic division. Thus a 'spectrum' implies not only a continual slight change in the type of bacteria, but also that the numbers of bacteria either remain constant for each type, or the numbers fall off slowly from some central type. The vast numbers of bacteria in the rhizosphere make measurement of the numbers of various types an extremely difficult task, liable to very large sampling errors. Conditions in the rhizosphere favour a large number of organisms from a great range of species, with common features such as being predominantly Gram-negative rods with a fast growth rate and the ability to rapidly utilize organic supplements (Lochhead, 1940; Rovira, 1956; Rouatt & Katznelson, 1957; Katznelson & Rouatt, 1957; Zagallo & Katznelson, 1957).

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RNA Synthesis and Degradation during Antibiotic Treatment and its Relation to Antibiotic-induced Lag

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SUMMARY

When growing cells of *Escherichia coli* were treated with chloramphenicol or erythromycin for 1 hr. and then suspended in antibiotic-free medium, there was a 45 min. lag before growth resumed. By eliminating growth factors or other essential nutrients during antibiotic treatment, it was possible to show that the lag occurred only when ribonucleic acid (RNA) synthesis could take place and did not require the synthesis of deoxyribonucleic acid (DNA). This antibiotic-induced RNA was apparently abnormal and was degraded when the antibiotic was removed. This degradation is a hydrolytic process and does not require the presence of a complete growth medium. During the recovery from the antibiotic-induced lag, DNA and protein synthesis did not occur, but RNA synthesis occurred, even though this new RNA synthesis was not required for the lag to be overcome. When antibiotic-treated cells were suspended in phosphate buffer, a decrease in optical density of the suspension occurred which resembled a lytic process, but lysis apparently did not occur. Although these results clarify considerably earlier observations on antibiotic-induced lag, they leave unsolved the question of why the antibiotic-induced RNA is abnormal, and how it brings about the lag.

INTRODUCTION

Several years ago there were independent reports from several laboratories of accumulation of RNA and its subsequent degradation in chloramphenicol-inhibited cells (Hahn *et al.* 1957; Neidhardt & Gros, 1957; Horowitz, Lombard & Chargaff, 1958). The first two groups of workers showed that cells which had been treated with antibiotic, and had accumulated RNA, exhibited a lag before growth resumed when they were suspended in antibiotic-free medium. Although these workers had indirect evidence for the relation of this antibiotic-accumulated RNA to the subsequent lag, they supplied no direct evidence for this hypothesis.

The present work was begun when the authors (Brock & Brock, 1959*a*) discovered that erythromycin and chloramphenicol were similar in action and began to compare a number of facets of erythromycin action with those of chloramphenicol. It was readily shown that erythromycin also brought about RNA accumulation, and erythromycin-treated cells also exhibited a lag when resuspended in antibiotic-free growth medium. The availability of a mutant of *Escherichia coli* 15 T-U⁻ (Barner &

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Cohen, 1958), which requires thymine + uracil for growth, provided us with a tool for controlling RNA and DNA synthesis during antibiotic treatment and recovery, to show directly the involvement of RNA in antibiotic-induced lag. A preliminary report of this work has been presented (Brock & Brock, 1960).

METHODS

Organisms. *Escherichia coli* strains 15 T-U⁻ and T-A-U⁻ were kindly provided by Dr S. Cohen; *E. coli* B₄ (prolineless) was obtained from Dr L. Frank, and *E. coli* ML35, from Dr J. Monod. A methionine-requiring mutant of strain 15 T-U⁻ was isolated by the authors and is designated T-U-M⁻.

Medium and growth conditions. A defined medium of the following composition was used (g.): K₂HPO₄, 6.0; KH₂PO₄, 3.0 NH₄Cl, 0.5; MgSO₄.7H₂O, 0.1; glucose, 4.0; distilled water to 1 l. Thymine and uracil were added to give final concentrations of 8 and 40 µg./ml. except in the radioactivity experiments. Arginine and methionine were added to final concentrations of 50 µg./ml., and proline to 100 µg./ml. except in the radioactivity experiments.

Growth was followed by measuring optical density (o.d.) at 420 mµ on a Lumetron colorimeter.

Organisms were grown overnight in defined medium at 37° on a rotary shaker, diluted into fresh medium to an optical density of 0.035–0.050, and dispensed 100 ml./500 ml. flask. After logarithmic growth had been re-established, at approximately o.d. 0.150, the suspensions were chilled and centrifuged, then washed twice with cold buffer of the following composition (g.): K₂HPO₄, 6.0; KH₂PO₄, 3.0; distilled water, 1 l. The organisms were then resuspended in fresh medium with and without various supplements at 37° to o.d. 0.150 and replaced on the shaker for antibiotic treatment. This same procedure for washing suspensions was repeated after the antibiotic treatment.

Antibiotics. Chloramphenicol was purchased from Parke, Davis and Co., and erythromycin was a gift from Eli Lilly and Co. Stock solutions of these antibiotics were prepared in 50% (v/v) aqueous ethanol, and these were diluted 1/100 upon addition to suspensions of organisms. Chloramphenicol was used at a final concentration of 50 µg./ml. and erythromycin at 1000 µg./ml.

Assays. For chemical assays of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein, 10 ml. samples were removed and chilled in an ice bath at 4°, centrifuged in the cold for 5 min. at 13,000 rev./min., and washed in succession with 8 ml. volumes of cold 0.5 N-HClO₄, 95% (v/v) ethanol in water, and anhydrous ether. The residue was then air dried and extracted with 4 ml. of 0.5 N-HClO₄ at 90° for 15 min. The extracts were allowed to cool to room temperature and were then centrifuged. A 1 ml. sample of this supernatant was used for assay of DNA by the method of Burton (1956). A 1 ml. sample was diluted with 2 ml. of 0.5 N-HClO₄ for assay of RNA by the method of Chargaff & Davidson (1955). For assay of protein by the method of Lowry (Lowry, Rosebrough, Farr & Randall, 1951) the residue from the hot HClO₄ extraction was suspended in 10 ml. of N-NaOH and heated to 100° for 1 hr.; a 1 ml. sample was used. Commercial preparations of DNA, RNA, and lysozyme were used as standards.

Radioactivity experiments. Thymine-2-¹⁴C (Volk Radiochemical Co., specific

activity 1.0 mc./mm) was added to a concentration of 2 $\mu\text{g./ml.}$, 1.15×10^4 c.p.m./ml. Uracil-2- ^{14}C (Volk Radiochemical Co., specific activity 3.2 mc./mm) was added to a concentration of 10 $\mu\text{g./ml.}$, 1.95×10^5 c.p.m./ml. Proline (Schwartz Bioresearch, Mt Vernon, New York; specific activity 100 $\mu\text{c./mm}$) was added to a concentration of 45 $\mu\text{g./ml.}$, 3.88×10^4 c.p.m./ml. Sodium sulphate- ^{35}S (Oak Ridge National Laboratories, carrier-free) was added at 8.64×10^4 c.p.m./ml.

For measurement of incorporation of the radioactive compounds, washed organisms were resuspended in the appropriate medium, the radioactive compound added, and samples taken. In the case of uracil, 0.1 ml. samples were taken, cooled in an ice bath, and 0.9 ml. of cold 0.5 N-HClO₄ was added. For thymine, proline and sulphate, 1.0 ml. samples were taken, cooled, and 9.0 ml. of cold 0.5 N-HClO₄ added. After standing for 30 min. in the cold, the suspensions were filtered on membrane filters (Millipore type HA, 0.45 μ), washed twice with equal volumes of cold 0.5 N-HClO₄, and the filter disks glued to stainless steel planchets (Roberts *et al.* 1957).

In experiments in which the RNA was labelled with radioactive uracil during or before antibiotic treatment, uracil of a specific activity of 0.96 mc./mm was added at a concentration of 10 $\mu\text{g./ml.}$ Samples (0.2 ml.) were taken and mixed with 1.8 ml. cold 0.5 N-HClO₄ and processed as above.

Counts were made with a Nuclear Chicago gas flow counter. No corrections for self-absorption were made. The results are reported as counts/min. above background.

β -Galactosidase assay. To 1.0 ml. samples of cell suspensions or supernatant fluids of ML 35 grown in salts + glycerol medium was added 4 ml. of M/600 *o*-nitrophenyl- β -D-galactoside (ONPG) in 0.1 M-Na₂HPO₄ buffer (pH 7). After incubation at 37° for sufficient colour development, the reaction was stopped by adding 5 ml. of M-Na₂CO₃. Following centrifugation the optical density at 420 m μ was determined; *o*-nitrophenol was used as a standard (Brock & Brock, 1959*b*).

Viable counts. Viable counts were made by the pour plate method with nutrient agar; counts were made after incubation for 24 hr.

RESULTS

Antibiotic-induced lag occurs only when RNA synthesis can take place

In the following work, chloramphenicol and erythromycin gave essentially the same results, so for simplicity results are presented for chloramphenicol only. When chloramphenicol (50 $\mu\text{g./ml.}$) or erythromycin (1000 $\mu\text{g./ml.}$) was added to growing cultures of *Escherichia coli*, protein synthesis was almost completely inhibited, while RNA and DNA synthesis continued. When the organisms were washed several times to remove antibiotic and then resuspended in growth medium, there was a lag of 45–60 min. before growth resumed. This lag did not occur when the organisms were not treated with antibiotic, nor did it occur when growth was inhibited by withholding an essential amino acid or other nutrient such as a nitrogen, carbon or phosphorus source, rather than by antibiotic inhibition. It was not due to the killing of a portion of the population. Since the RNA synthesized in the presence of chloramphenicol differs in a number of ways from normal RNA (Horowitz *et al.*

1958; Pardee *et al.* 1957), it seemed possible that the lag was due to the prior synthesis of this abnormal RNA (Hahn *et al.* 1957). To test this point, a mutant of *E. coli* was used which requires thymine + uracil for growth, since Barner & Cohen (1958) had shown that withholding uracil prevented RNA synthesis without preventing DNA synthesis, and withholding thymine prevented DNA synthesis without affecting RNA synthesis. When growing cultures of this mutant were treated with antibiotic for 2 hr. in the presence or absence of either of these growth factors, and then tested for lag by resuspending them in antibiotic-free medium containing both growth factors, the following results were obtained: (1) With thymine + uracil present during antibiotic treatment there was a 1 hr. lag on recovery. (2) With thymine only present and uracil absent there was no lag. (3) With uracil only present and thymine absent there was a 1 hr. lag on recovery. (4) With neither thymine nor uracil present there was no lag. RNA synthesis did not take place in the absence of uracil, and DNA synthesis did not take place in the absence of thymine. These results indicate that, for the subsequent lag to occur, the cells must be able to synthesize RNA during antibiotic treatment.

As a further test of this point, uracil was added or withheld during the first or second hour of antibiotic treatment, or during both hours, or neither, and the amount of RNA synthesized was measured and correlated with the subsequent lag. These results are shown in Table 1, in which the lag is given as the length of time

Table 1. *Synthesis of RNA and induction of lag during chloramphenicol treatment in the presence or absence of uracil*

Escherichia coli 15 T⁻U⁻, in salts + glucose medium, thymine and chloramphenicol present in all.

	RNA* synthesized during chloramphenicol treatment (μ g./ml.)	Lag† (min.)
No uracil	0	0
Uracil, 2 hr.	46	55
Uracil, 1st hr.	33	45
Uracil, 2nd hr.	38	30

* Initial RNA, 35 μ g./ml.

† Lag measured after reincubation in salts + glucose + thymine and uracil, given as time for o.d. to double minus the time for o.d. of logarithmically growing cells to double.

for the culture to double in optical density after resuspension, after subtracting the doubling time of a logarithmically growing culture (Lockhart, 1960). It can be seen that the lag was longer when uracil was present for 2 hr. than when it was present for 1 hr. Further, uracil present during the first hour induced a longer lag than uracil present during the second hour, even though approximately equal amounts of RNA were synthesized under both conditions. DNA synthesis occurred in all four treatments.

As a further correlation between RNA synthesis and lag, two mutants were used which required amino acids in addition to thymine and uracil. One of these was *Escherichia coli* 15 T⁻A⁻U⁻ of Kanazir, Barner, Flaks & Cohen (1959) which was isolated by them from *E. coli* 15 T⁻ by an apparent one-step mutation to both

arginine and uracil requirements, although the blockage in synthesis of both of these compounds is not complete. When arginine was withheld in the presence of uracil and thymine, no RNA synthesis occurred, apparently because of the obligatory coupling of RNA and protein synthesis under these conditions. However, when chloramphenicol or erythromycin was present, RNA synthesis occurred in the absence of arginine, even in arginine-starved cells. This is at variance with the observations of Pardee & Prestidge (1956) and of Gros & Gros (1958) that RNA synthesis did not occur in the absence of an essential amino acid, even in the presence of chloramphenicol. However, since this *E. coli* mutant T-A-U⁻ can form some arginine, this may be enough to support the synthesis of antibiotic-induced RNA even though it will not support normal RNA synthesis. When growing organisms of this mutant were treated with antibiotic in the absence of arginine, a 45 min. lag was induced, while 21 µg./ml. of RNA were synthesized (Table 2). When both arginine and uracil were withheld, there was no antibiotic-induced RNA synthesis and no lag. This is further support for the idea that antibiotic-induced lag is due to the synthesis of abnormal RNA, since in this mutant, in the absence of arginine, only antibiotic-induced RNA can accumulate, and this accumulation leads to a subsequent lag.

A methionine-requiring mutant of *Escherichia coli* 15 T-U⁻, designated T-U-M⁻,

Table 2. *Synthesis of RNA and induction of lag during chloramphenicol treatment; arginine and uracil present or absent during antibiotic treatment*

Escherichia coli 15 T-A-U⁻, in salts + glucose medium, thymine present in all during treatment; thymine, arginine and uracil present in all during recovery.

Conditions during treatment	RNA* synthesized during treatment (µg./ml.)	Lag† (min.)
Arginine + uracil	55	0
Arginine + uracil + chloramphenicol	21	45
Uracil, no arginine	3	0
Uracil + chloramphenicol, no arginine	21	45
No uracil, no arginine	0	0
No uracil, no arginine, chloramphenicol present	2	0

* Initial RNA, 35 µg./ml.

† Generation time of controls, 65 min.

Table 3. *Synthesis of RNA and induction of lag during chloramphenicol treatment; methionine present or absent during antibiotic treatment*

Escherichia coli 15 T-U-M⁻, in salts + glucose medium, thymine and uracil present in all during treatment; thymine, uracil and methionine present in all during recovery.

Conditions during treatment	RNA* synthesized during treatment (µg./ml.)	Lag† (min.)
Methionine	93	0
Methionine + chloramphenicol	24	30
No methionine	7	0
No methionine, chloramphenicol present	40	40

* Initial RNA, 58 µg./ml.

† Generation time of controls, 70 min.

was isolated by the authors. This mutant could also synthesize some RNA in the absence of methionine, when chloramphenicol was present, and a lag was found on recovery (Table 3). A proline-requiring mutant, *E. coli* B₄, did not synthesize antibiotic-induced RNA in the absence of proline and did not exhibit any antibiotic-induced lag. It can therefore be concluded that antibiotic-induced lag occurs only when antibiotic-induced RNA is synthesized, and the lag may be related to the synthesis of some abnormal RNA. The results with the arginine- and methionine-requiring mutants show clearly that it is an antibiotic-induced RNA which is responsible for the lag, and not an excess of a normal RNA.

Ability to synthesize new RNA is not a prerequisite to recovery from antibiotic-induced lag

Growing cultures of *Escherichia coli* 15 T-U⁻ were treated with antibiotic for 1 hr. in glucose salts medium with thymine + uracil present. The organisms were then washed and resuspended in antibiotic-free medium from which uracil, thymine, glucose, ammonium chloride or phosphate was omitted. Ordinarily, organisms treated as above and resuspended in complete medium began to divide only after incubation for 45 min. The requirement for various nutrients during this lag period was determined by withholding the nutrient for 45 min., then adding it back, and determining whether the organisms started growing immediately (i.e. with no additional lag) or exhibited a further lag before growing. In each experiment, a control flask of antibiotic-treated organisms had the nutrient present during the lag. The lag was not increased in length significantly by withholding any of the nutrients essential for the synthesis of RNA, DNA or protein, namely, glucose, phosphate, ammonium salt, uracil or thymine. Since normal organisms do not grow or synthesize macromolecules when any of these nutrients is withheld, this seems to indicate that recovery from the antibiotic-induced lag is essentially a degradative process, probably involving the destruction of abnormal RNA. Since the degradative enzymes can operate in non-growing organisms (Horiuchi, 1959; Mandelstam, 1958), this interpretation seems justified. It also seems unlikely that the lag is due to a deficiency in any of the small molecules produced by the cell, since the addition of 0.5% (w/v) yeast extract during the recovery period did not shorten the lag.

Synthesis of DNA and protein does not begin until the lag is over; but RNA synthesis can begin immediately

Preliminary measurements by chemical methods of RNA, DNA and protein during the recovery period revealed that synthesis of these macromolecules did not begin until the lag was over. However, chemical measurements would not indicate any turnover of macromolecules, such as degradation and resynthesis. Therefore, radio-isotope methods were used to follow these processes. Since the organism used, *Escherichia coli* 15 T-U⁻, has absolute requirements for both thymine and uracil, it was possible to measure synthesis of DNA and RNA by determining the incorporation of radioactive thymine and uracil into cold perchloric acid-insoluble material in whole organisms. Preliminary experiments comparing the isotope method with the chemical method indicated a close correspondence when logarithmically growing cultures were used. Presumably none of the radioactive thymine enters RNA, and

only a small fraction of the radioactive uracil enters DNA, by way of cytosine. In every experiment a no-glucose control used to check for non-specific absorption always gave a negligible reading. As a measure of protein synthesis, the incorporation of ^{14}C -proline and $\text{Na}_2^{35}\text{SO}_4$ was determined.

Figure 1 shows the results of the incorporation studies with these isotopes. With thymine, proline or sulphate, there was a lag in the incorporation of isotope in chloramphenicol-treated organisms, and this lag paralleled the lag in growth. However, incorporation of ^{14}C -uracil differed from the other isotopes in one important

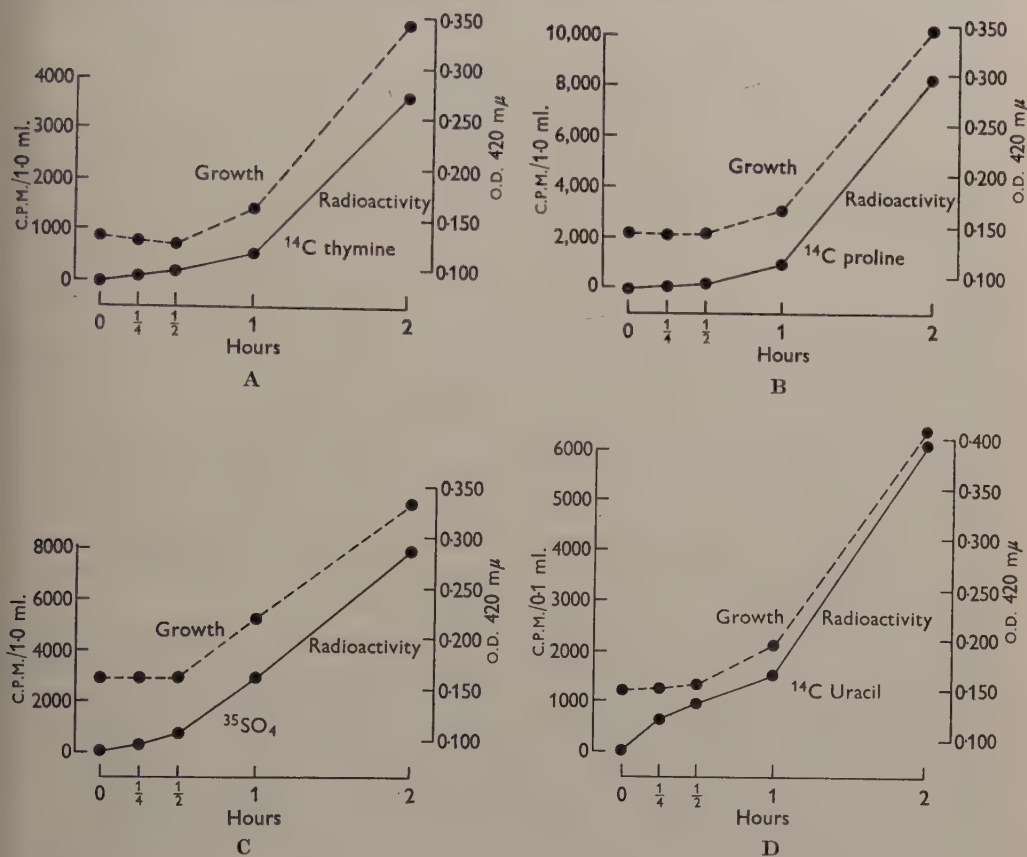


Fig. 1. Growth of *E. coli* 15 T-U- and incorporation of: (A) radioactive thymine, (B) radioactive proline, (C) radioactive sulphate, and (D) radioactive uracil during chloramphenicol-induced lag. ----, Growth; —, radioactivity.

respect. There was an initial rapid increase in uracil incorporation, followed then by a slower rate, and this was followed by the normal rate of incorporation after growth had resumed. Although brief, this initial rapid increase was reproducible. The interpretation is advanced that the antibiotic-treated organisms, although unable to synthesize DNA or protein during the lag, were able to synthesize RNA, and that the initial rapid increase in incorporation was due to synthesis of new RNA. However, during the antibiotic treatment the organisms have accumulated RNA which is being degraded, and soon this degradation provides sufficient non-radio-

active RNA precursors to dilute out the isotopic uracil, and the synthesis of new RNA probably then occurs predominantly from the non-radioactive sources. Only after all of this RNA was degraded, and the lag was overcome, was radioactive uracil again incorporated at a maximum rate. This interpretation supports the idea advanced by others (Kjelgaard, Maaløe & Schaechter, 1958) that when there is a shift in growth rate from low to high, RNA synthesis occurs in advance of this shift and apparently provides the higher concentrations of RNA necessary in the cell to support this new growth rate. It is concluded therefore that the synthesis of RNA can begin immediately in the non-growing cells, whereas DNA and protein synthesis can occur only after the lag is overcome.

*Loss of antibiotic-accumulated RNA may occur under
some conditions*

In earlier work on antibiotic-induced lag it was reported (Hahn *et al.* 1957; Neidhart & Gros, 1957; Horowitz *et al.* 1958) that the RNA which accumulated was degraded and excreted before growth resumed. It was suggested by these workers that this excretion was essential to the recovery process. In the present experiments, when the antibiotic-treated organisms were resuspended in complete growth medium, there was no evidence that the degradation of RNA was followed by the excretion of RNA precursors during the lag. However, when the organisms were suspended in non-growth medium, such as phosphate buffer, there was a marked loss of RNA from the cells during the recovery period. This observation is not completely at variance with earlier work, since Neidhardt & Gros (1957) and Horowitz *et al.* (1958) only studied excretion under non-growing conditions, and Hahn *et al.* (1957) used much longer periods of antibiotic treatment which may have brought about more drastic metabolic derangements. Horiuchi, Sunakawa & Mizuno (1958) also found in *Escherichia coli* B that antibiotic-induced RNA was excreted under non-growing conditions but not under growing conditions.

In the present work it was noted that whenever there was a loss in RNA there was a sharp decrease in optical density. This decrease in optical density seemed to indicate that antibiotic-treated organisms were lysing when they were not growing and not lysing when they were growing, and that excretion of RNA degradation products was not occurring, but that a complete lysis and release of all of the cell contents occurred in some of the organisms. More detailed studies showed this idea to be wrong; the results to be presented below show that antibiotic-induced RNA was degraded and the degradation products excreted under non-growing conditions, whereas under growing conditions they were immediately incorporated into new RNA. The decrease in optical density was not due to lysis but to some other change in the morphology of the cells.

It was found that the membrane filter technique did not distinguish between high-molecular weight RNA within cells or in lysates. Thus, when cells were labelled with ^{14}C -uracil in the absence of antibiotic, lysed with lysozyme and ethylene diamine tetracetic acid, and the lysates diluted into cold perchloric acid and filtered, all of the radioactivity was retained by the filter, and the counts obtained were the same as when unlysed organisms were used. Treatment of lysates for 30 min. with 0.1 N-NaOH at 0° almost completely degraded the RNA of these lysates, so that the radioactivity then passed through the filter. These results showed that the mem-

brane filter technique would withhold high molecular weight RNA whether within cells or free, whereas low molecular weight materials passed through the filter.

Two series of experiments were set up. In the first, the RNA of cells was labelled by growth for three generations in radioactive uracil in the absence of chloramphenicol. The organisms were then washed and suspended in growth medium with chloramphenicol and cold uracil for 1 hr., then washed again to remove antibiotic and suspended in phosphate buffer or growth medium with antibiotic. The optical density of the suspension was measured periodically, and samples were taken for membrane filtration. It can be seen in Fig. 2*a* that in growth medium there was the usual lag on recovery but no loss of radioactivity. In phosphate buffer there was a marked decrease in optical density in the suspension of antibiotic-treated organisms but no loss in radioactivity. This shows that preformed RNA was not degraded during recovery, although the decrease in optical density seems to indicate some antibiotic-induced change in the organisms.

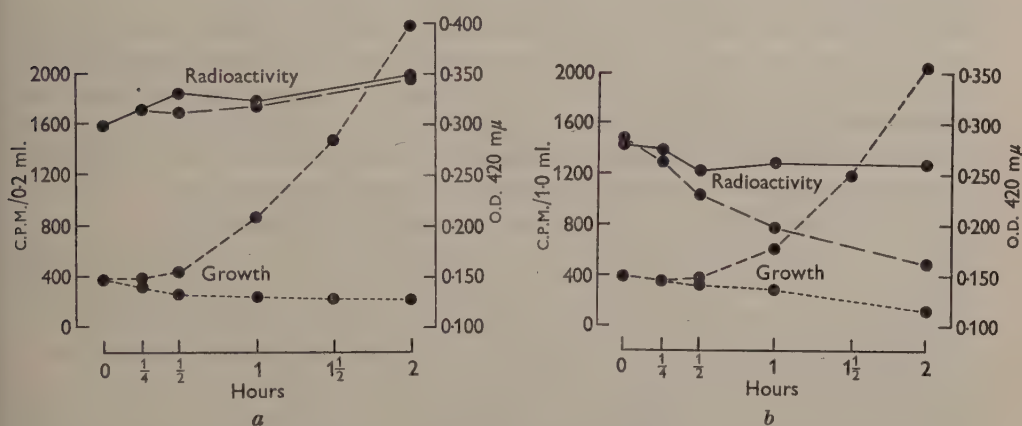


Fig. 2. Growth of *Escherichia coli* 15 T-U- and changes in radioactivity after treatment with chloramphenicol. (a) Cells labelled with ¹⁴C-uracil before chloramphenicol treatment, then 1 hr. chloramphenicol treatment, then recovery in antibiotic-free salts + glucose medium or phosphate buffer. (b) Cells labelled with ¹⁴C-uracil during 1 hr. chloramphenicol treatment, then recovery in antibiotic-free salts + glucose medium, or phosphate buffer. Graphs represent growth and retention or loss of radioactivity. —, Radioactivity, cells suspended in salts + glucose; — —, radioactivity, cells suspended in phosphate buffer; - - - -, growth, cells suspended in salts + glucose; - - - - -, growth, cells suspended in phosphate buffer.

In the second series of experiments, the RNA was labelled by having ¹⁴C-uracil present during a 1 hr. chloramphenicol treatment and the organisms then washed and resuspended in growth medium or in phosphate buffer and the optical density and loss of radioactivity determined. In Fig. 2*b* it can be seen that in growth medium there was the usual lag on recovery but only a slight and transitory loss in RNA. In phosphate buffer there was a definite decrease in optical density and a marked loss of radioactivity. These results show that antibiotic-accumulated RNA was degraded to low molecular weight material and lost from the organisms when they were suspended in non-growing conditions. Under growing conditions there was only a negligible loss in radioactivity, so the degraded material must have been immediately incorporated into new RNA. This immediate re-incorporation is in

agreement with the results above which showed that RNA synthesis began immediately, even during the lag period. Thus antibiotic-accumulated RNA was degraded during recovery, whereas normal RNA was not degraded even in antibiotic-treated organisms.

The decrease in optical density of suspensions of antibiotic-treated organisms in phosphate buffer seemed to indicate that lysis occurred in these organisms. However, viable counts of control and antibiotic-treated organisms of samples taken at intervals up to 2 hr. in phosphate buffer indicated no loss in viability which would be expected to accompany lysis (Table 4). As a more sensitive measure of possible lysis the hydrolysis of ONPG by a β -galactosidase-constitutive, permease-less mutant of *Escherichia coli* (ML35) was measured (Brock & Brock, 1959b). In addition, the enzyme activity of supernatant fluids from such organisms was measured. No increase in rate of hydrolysis was noted in antibiotic-treated organisms as compared to control organisms after 1 hr. in phosphate buffer, although most of the radioactivity was lost during this 1 hr. treatment, as shown in Fig. 2b. Thus there was no evidence that the decrease in optical density was due to frank lysis, or to a markedly altered permeability of the cells brought about by the antibiotic treatment. An alternative explanation is that the decrease in optical density was due to an increase in pool materials because of an accumulation of RNA degradation products. This would result in an increased osmotic pressure in the cell, bringing about an increase in diffusion of water into the cells, leading to a swelling of the cells and a decrease in optical density. This latter explanation seems the more likely.

Table 4. *Viable counts*

Escherichia coli 15 T-U⁻, previously treated for 1 hr. with chloramphenicol in salts + glucose + thymine + uracil, resuspended in phosphate buffer and viable counts determined during incubation at 37°.

Time in buffer	Viable count $\times 10^6$	O.D.
0	263	145
30 min.	262	133
1 hr.	243	130
2 hr.	247	120

DISCUSSION

The results of the present work help to clarify the problem of antibiotic-induced lag and antibiotic-induced RNA synthesis in *Escherichia coli*. It appears that the lag occurs only under conditions where antibiotic-induced RNA synthesis occurs, and when this antibiotic-induced RNA synthesis is prevented by any means (such as deprivation of a nutrient essential for its synthesis) no subsequent lag occurs. Interesting in this regard are the results with the arginine- and methionine-requiring mutants which did not synthesize normal RNA but which synthesized antibiotic-induced RNA in the absence of added arginine and methionine, respectively, and which exhibited a subsequent lag after this synthesis. A proline-requiring mutant which did not synthesize chloramphenicol-induced RNA in the absence of proline exhibited no lag on recovery.

The observations of Borek & Ryan (1958) are pertinent to this discussion. They found that in one particular methionine-requiring mutant of *Escherichia coli*, RNA

was synthesized in the absence of methionine, and the RNA-rich organisms exhibited a lag after methionine was added before growth was resumed. (No antibiotic was added in this experiment.) This lag may have been due to the accumulation of abnormal RNA which had to be degraded before growth could be resumed. The results of Borek & Ryan may be analogous to the present results concerning antibiotic-induced lag.

Since no requirements for energy-yielding or essential nutrients were found, recovery from the antibiotic-induced lag appears to be a degradative process in which hydrolytic enzymes cleave abnormal RNA. During the recovery period, DNA and protein synthesis did not occur, but the organisms appeared to be able to synthesize RNA during the lag while RNA was being degraded, provided that energy sources and the RNA precursors were supplied. This new RNA synthesis is probably not essential for recovery, since recovery occurred in the absence of many nutrients essential for new RNA synthesis. However, the fact that RNA synthesis occurred during the lag (even though not required) may be a further indication of the hierarchy of macromolecular syntheses for growing organisms (Kjelgaard *et al.* 1958).

Excretion of the degradation products is not a requirement for recovery, since when the cells were suspended in growth medium, these degradation products were immediately incorporated into new high molecular weight RNA. The degradation products apparently accumulated both within the cell and in the supernatant fluid under non-growth conditions (Neidhardt & Gros, 1957). The accumulation within the organisms was probably responsible for the decrease in optical density of suspensions that took place in organisms under non-growth conditions, since there was no evidence of lysis.

An unanswered question from this and earlier investigations is: why does the lag occur? It is not a sufficient explanation to state that this is abnormal RNA and thus prevents growth. Why is this RNA abnormal? As yet there is no answer to this question, since so much is lacking about our understanding of the macromolecular strategy of normal growth processes.

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Some Properties of a Cytopathogenic Bovine Orphan Virus (Van den Ende Strain)

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SUMMARY

A cytopathogenic bovine orphan (CBO) virus isolated from a bovine with lumpy skin disease appears unrelated to any of the three groups of viruses now associated with this condition. The virus multiplies efficiently in cultures of whole chick embryo tissue and may be titrated by plaque counting methods. Tissue culture fluids contain infective particles of at least three sizes, with sedimentation constants of 460, 72 and 20 S. The intermediate particle, if spherical and lipid free, would be comparable in size to the virus of foot and mouth disease. Heterogeneity of the virus has also been demonstrated by plaque morphology, electrophoresis and chromatography on DEAE cellulose. It is adsorbed to fresh but not to receptor-destroying enzyme (RDE) treated red cells of chicken and goose, but haemagglutination has not been observed with cells of any of the species tested. The virus lacks enzyme activity comparable to that of influenza virus.

INTRODUCTION

While investigating the etiology of bovine lumpy skin disease, Van den Ende, Alexander, Don & Kipps (1948) and Van den Ende, Don & Kipps (1949) reported the isolation in eggs of a virus from a skin nodule and a lymph gland of a calf which had died while the disease was still active. This virus produced unique and characteristic lesions in chick embryos. By common usage and in affection for the late Director of the Virus Research Unit, this virus has come to be known throughout the Union of South Africa as the 'Van den Ende Virus' but it is better called the Van den Ende strain of a cytopathogenic bovine orphan (CBO) virus. What appeared to be a successful transmission of the disease to bovines by inoculation of material from the 66th egg passage (Van den Ende & Turner, 1950), led to the assumption that this virus was in fact the cause of lumpy skin disease (Polson & Turner, 1954). Further studies, however, did not confirm this association and the earlier apparently successful transmission was recognized in retrospect as a natural infection in the laboratory herd.

The etiology of lumpy skin disease is now well known, and Alexander, Plowright & Haig (1957) reported the isolation of three groups of viruses from bovines suffering from the disease; of these viruses the prototypes Allerton virus and Neethling virus are representative of the responsible etiological agents. Although numerous isolations of these two viruses have been made, neither of them bears any relationship to the virus described in this paper. Attempts to re-isolate CBO virus from

bovines with lumpy skin disease have all been unsuccessful. An enteric cytopathogenic bovine orphan (ECBO) virus isolated during this investigation was found to possess some of the characters of the Van den Ende strain but was antigenically distinct. The present paper describes some of the physical and biological properties of the Van den Ende strain of CBO virus. The effect of inhibitors present in normal animal sera upon this virus is discussed in another communication (Turner, Kipps, Polson & Van den Ende, 1961).

METHODS

Virus

After its isolation, CBO virus was at first maintained by periodic amniotic or allantoic passage in chick embryos. Later it was found to be stable after drying from the frozen state, and active lyophilized reference stocks of the 16th egg passage have been kept for many years.

Propagation in tissue culture

CBO virus grows readily in cultures of whole chick embryo tissue. Cell suspensions were prepared by tryptic digestion of 9–10 day chick embryos and grown in a medium consisting of 0.5% (w/v) lactalbumin hydrolysate in Hanks balanced salt solution with 20% (v/v) horse serum. In the maintenance medium the serum was omitted.

Virus stocks for the majority of the experiments were harvested from Roux flask cultures when cytopathic changes were complete, approximately 3 days after inoculation. After light centrifugation to remove tissue debris, the supernatant fluid was stored in sealed glass ampoules at -70° . There was little loss of infectivity over a period of many months.

Titration

The titration of the virus in eggs has been described (Van den Ende & Turner, 1950). Titrations in tissue culture were carried out by the plaque techniques of Dulbecco (1952) and Cooper (1955). Chick embryo cultures were prepared in 4 cm. diameter Petri dishes using not less than 1×10^7 cells/dish in 3 ml. nutrient medium and duplicate dishes were seeded with 0.2 ml. volumes of virus suspension or serum-virus mixture. The plates were incubated at 37° in air containing about 5% (v/v) CO_2 for 2 days, after which they were flooded with 1/5000 neutral red in Earle's saline. The dye was removed after 2 hr. when the plaques could be easily counted. Repeat counts were made next day. Titres were expressed as plaque forming units (p.f.u.).

Antisera

Immune sera were produced in fowls and rabbits. Adult hens were inoculated with graded intramuscular doses of emulsions of infected embryos. The intravenous route was used for the rabbits. Blood was collected 10–12 days after the last of a course of 7–8 injections given twice weekly. The blood was allowed to clot and the separated serum heated at 56° for 30 min. and stored at -20° .

Neutralization tests

Two methods were used. In the first, a single concentration of virus known to produce about 60 plaques/plate was used. Unit volumes of tenfold dilutions of serum were mixed with equal volumes of the virus dilution, the mixtures allowed to stand at room temperature for 1 hr. and each mixture then used to inoculate duplicate agar suspension cultures of chick embryo cells (Cooper, 1955).

In the second method serial tenfold dilutions of both serum and virus were used so that the titre of the virus in the presence of different concentrations of antibody or inhibitor could be measured. The time of contact and the method of plating were the same as in the method employing a single concentration of virus.

Zone electrophoresis

The technique of Polson & Cramer (1958) was used. The virus suspension was mixed with phenol red and rabbit haemoglobin as reference substances. Electrophoresis was allowed to proceed at room temperature under a voltage gradient of 3.5 V./cm. and a current of 15 mA. through a sucrose density gradient (35 to 0 %) in borate buffer (pH 8.6) until the phenol red reached a level of 16 cm. from the origin. The gradient column between the origin and the phenol red was divided into 16 equal fractions by removal through a fine capillary at the bottom of the column. Each fraction was titrated for infectivity in agar suspension cultures of chick embryo cells (Cooper, 1955).

Ultracentrifugation

Sedimentation coefficients were determined by the methods of Polson & Van Regenmortel (to be published) using the S.W. 39 rotor of the model LH Spinco centrifuge at temperatures below 4°. In these methods convection was prevented by a steep sucrose concentration gradient below the layer of virus suspension. In a series of tubes the length of the 'effective virus column' was kept constant at 1 cm. After centrifugation the whole of the fluid above the sucrose gradient was removed and titrated. In this way the conditions of centrifugation necessary to cause sedimentation of the virus through a measured distance were accurately maintained. Series of tubes prepared as indicated were centrifuged either at constant rotor velocity for different time intervals, or for constant periods of time at various rotor velocities.

The sedimentation constants of the particles under investigation were calculated from the following equations:

$$S_{20} = 3.50 \frac{\eta_T}{\eta_{20}} \frac{\log X}{N^2 t}, \quad (1)$$

where η_T and η_{20} are the viscosities of the dispersion medium at the temperature of centrifugation and of water at 20° respectively, N the rotor velocity in rev./min. and t the time of centrifugation in minutes,

$$X = \frac{x+l}{x+l(C_t/C_0)}$$

in which x is the distance from the upper meniscus to the centre of rotation, l is the effective column (i.e. the distance between the upper meniscus and the upper limit

of the sucrose gradient), and C_t/C_0 the ratio of average virus or protein concentration in the 'effective column' after and before ultra-centrifugation.

DEAE chromatography

The CBO virus in infected tissue culture fluid was concentrated by pervaporation and centrifugation at 30,000 rev./min. for 2 hr. The final pellet was suspended in 2 ml. distilled water and applied to a 10 cm. column of DEAE cellulose (Peterson & Sober, 1956), containing 0.7 g. of adsorbent which had been equilibrated with 0.01 M-phosphate buffer (pH 7.2). A gradient prepared from 50 ml. of the buffer and 50 ml. of M-NaCl was used for elution. Twenty-five 2 ml. fractions were collected and titrated individually in chick embryo tissue culture by the plaque technique.

Enzymes

Dried commercial receptor-destroying enzyme (RDE; Philips-Roxane) was reconstituted to the volume recommended by the manufacturer in pH 6.0 buffer containing 100 units penicillin and 100 µg. streptomycin/ml. Active influenza B virus (Lee) propagated in eggs according to well known standard techniques was used as a source of neuraminidase.

RESULTS

Adaptation of virus to tissue culture

Virus passaged in chick embryos grew well in chick embryo tissue cultures and produced plaques. The ratio of p.f.u./ml. to the titre in chick embryo was relatively low in extracts of infected embryos and in fluid obtained from the first two or three passages in tissue culture, but the ratio increased rapidly to a value which remained almost constant after many passages in tissue culture (Table 1). The typical lesions are still regularly observed in chick embryos inoculated with the tissue culture adapted virus.

Table 1. *The adaptation of CBO virus to chick embryo tissue culture and the ratio of p.f.u./ml. to the titre in eggs*

Virus	p.f.u./ml.	Titre in eggs (LD 50/ml.)
Suspension of virus-infected egg	$10^{3.2}$	$10^{6.2}$
Suspension from plate tissue culture after 1 TC passage	$10^{4.4}$	$10^{7.3}$
Suspension from plate TC after 3 TC passages	$10^{6.1}$	$10^{7.3}$
Suspension from plate TC after 4 TC passages	$10^{6.2}$	$10^{7.1}$

Plaque morphology

After incubation at 37° for about 48 hr. two types of plaque were encountered. One was well defined and 1–2 mm. in diameter while the other, appearing in roughly equal numbers, was less well defined and measured 2–5 mm. in diameter. Incubation for another 24 hr. resulted in the enlargement of existing plaques and the appearance of many secondary plaques. On subculture, virus from either type of plaque yielded a mixture of the two.

Neutralization tests

The concentration of the virus clearly has an important bearing on the apparent neutralizing titre of the serum (Table 2). Most CBO fowl immune sera in dilutions of 10^{-3} showed significant neutralization. These sera did not neutralize the Allerton and Neethling strains of lumpy skin disease virus.

Titration of antibodies to CBO virus in bovine sera of both normal animals and those convalescent from lumpy skin disease were found to be unreliable owing to the non-specific neutralization by inhibitors in both groups of sera. Neutralization tests with CBO antisera produced in rabbits were similarly complicated by the presence of high titre non-specific inhibitors. These inhibitors were found in many other animal sera in varying concentration (Turner *et al.* 1961).

Table 2. *Neutralization of the virus by fowl immune serum in agar suspension cultures of chick embryo cells*

c, confluent plaques; sc, semi-confluent plaques.

Virus dilution	Average number of plaques/plate serum dilution					Control without serum
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
10^{-2}	0	12	c	—	—	c
10^{-3}	0	1	58	c	—	c
10^{-4}	0	0	35	sc	sc	c
10^{-5}	0	0	0	22	37	37
10^{-6}	0	0	1	3	6	5

Animal inoculation

Numerous attempts were made to develop a pathogenicity test in animals but the CBO-virus consistently failed to cause detectable symptoms or lesions when inoculated by various routes into rabbits, guinea pigs, adult and suckling mice, ferrets, sheep and bovines, as well as young chicks and adult domestic fowls.

Electrophoretic mobility of CBO virus particles

Three different electrophoresis experiments were done (Fig. 1). The two peaks indicate that the infective particles are electrophoretically inhomogeneous. These diagrams are quite different from those obtained by the same technique from the three groups of viruses isolated by Alexander *et al.* (1957) from bovines with lumpy skin disease (Polson, unpublished). No further characters have been ascribed to these two electrophoretically distinct components of the CBO virus.

Ultracentrifugation

Five experiments were done to determine the relationship between virus titre and the speed of centrifugation. The results (Fig. 2) show the presence in infected tissue culture fluid of virus particles having sedimentation constants of 460 S and 72 S. Experiments with fluid from which the 460 S particles had been removed by centrifugation at 10,000 rev./min. for 90 min. revealed, in addition to the 72 S particles, a small proportion of very light infective units which were not completely sedi-

mented during prolonged centrifugation at 30,000 rev./min. and had a sedimentation constant less than 20 S.

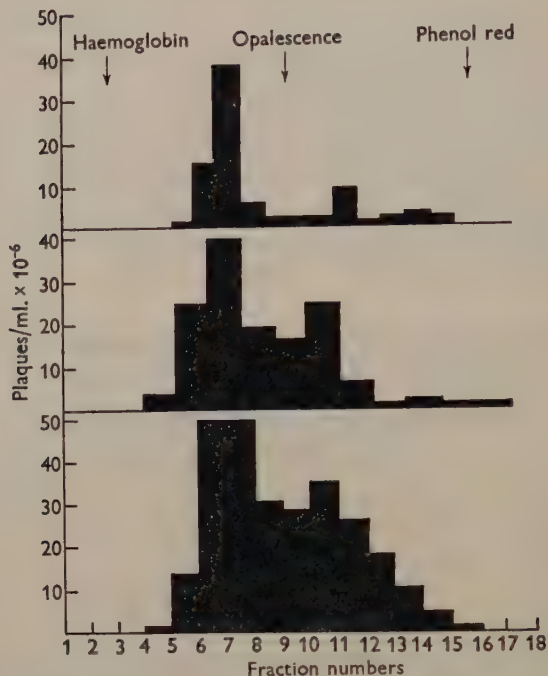


Fig. 1. Zone electrophoretograms of CBO virus in a sucrose density gradient at pH 8.6, 3.5 V./cm. and 15 mA.; phenol red and rabbit haemoglobin were added to indicate the progress of electrophoresis. The two peaks indicate inhomogeneity of the virus.

Chromatography on DEAE cellulose

Most of the virus was eluted at NaCl molarities between 0.2–0.3 (Fig. 3). About 10% of the virus appeared to be eluted at a slightly lower salt concentration than the component in the main peak.

Adsorption of CBO virus to red cells

Attempts were made to agglutinate various washed red cells with virus obtained from chick embryo tissue culture. These experiments were conducted at 4°, at room temperature and at 37° in a range of pH values from 6.0 to 7.6. All were negative. Virus suspensions concentrated by centrifugation to a titre of 4×10^8 p.f.u./0.2 ml. failed to agglutinate fowl and goose red cells even when followed by CBO fowl antiserum. However, adsorption of the virus by goose and fowl red cells was indicated by centrifuging mixtures of cells and virus after 3 hr. contact at 4° and titrating the supernatant fluid and the lysed sediment brought to the original volume (Table 3). The virus was eluted when the cells were lysed in distilled water. Since many normal sera were known to contain inhibitors capable of neutralizing the CBO virus, the effects of enzymes on the sites of adsorption of the virus particles on the red cells were tested. RDE effectively decreased the number of receptor sites on fowl cells for the CBO virus (Table 4). The virus itself, however, lacked enzyme activity of the

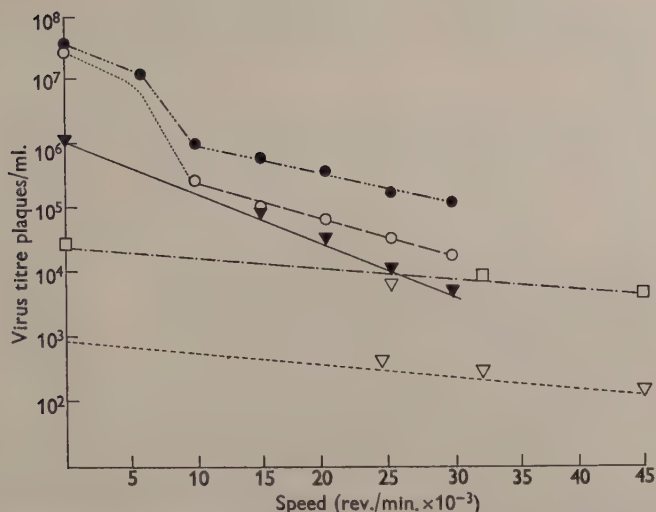


Fig. 2. Sedimentation diagrams of CBO virus. The infective titre of the fluid in the 1 cm. column above a level 7.1 cm. from the centre of rotation is plotted against the speed of centrifugation. The duration of centrifugation was 90 min. in all cases. ●—·····●, Titre of original virus suspension spun at the speeds indicated and showing the presence of rapidly and slowly sedimenting components; ○—·····○, repeat of first experiment (dotted line imaginary); ▼—▼, titre of virus suspension after centrifugation of the material from which the rapidly sedimenting components had been removed by 10,000 rev./min. for 90 min.; □—·····□, titre of virus suspension after centrifugation of the material from which the fast and the slower sedimenting components had been removed by 30,000 rev./min. for 90 min.; ▽—---▽, titre of virus suspension after centrifugation at 25,000 rev./min. for 90 min. to remove the particle of 20S = 72 and spun again at the velocities indicated.

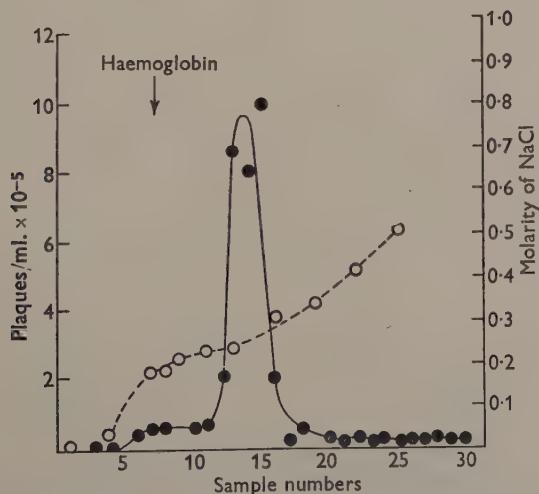


Fig. 3. Chromatography of CBO virus on DEAE cellulose. The bulk of the virus is eluted at concentrations between 0.2–0.3 M-NaCl, but about 10% of the virus elutes at slightly lower salt concentrations. The arrow marks position at which rabbit haemaglobin elutes. ●, Virus titre; ○, molarity of NaCl.

type exhibited by influenza virus, since adsorbed CBO virus did not elute at 37° and treatment of fowl cells with CBO virus did not reduce their agglutinability by influenza B virus (Lee).

Table 3. *The adsorption of CBO virus to goose and fowl cells*

Adsorption 3 hr. at 4°; 2 ml. packed cells and 0.5 ml. virus suspension.

Sample	Virus titre (p.f.u./ml.)
(1) Original untreated virus suspension	2.0×10^6
(2) Virus suspension after adsorption with goose RBC's	8.0×10^4
(3) Laked goose RBC's used for adsorption	3.5×10^6
(4) Virus suspension after adsorption with fowl RBC's	1.7×10^6
(5) Laked fowl RBC's used for adsorption	5.0×10^6

Table 4. *Adsorption of CBO virus to fowl red cells after treatment of cells with RDE*

2 ml. packed cells + 10 ml. RDE at 37° for 2 hr. washed $\times 5$ in saline then 2 ml. packed cells + 0.5 ml. virus; 3 hr. at 4°.

Sample	Virus titre (p.f.u./ml.)
(1) Original untreated virus suspension	4.0×10^6
(2) Virus suspension after adsorption with buffer treated cells	2.0×10^6
(3) Virus suspension after adsorption with RDE treated cells	6.0×10^6

DISCUSSION

The findings of Alexander *et al.* (1957) make it improbable that the CBO virus has any etiological relationship to bovine lumpy skin disease. This virus, isolated from a skin nodule of a calf in the acute stages of the disease, produced characteristic lesions in chick embryos which have been previously described (Van den Ende *et al.* 1948, 1949). CBO virus was readily adapted to growth in cultures of chick embryo cells, and produced well defined plaques in agar suspension cultures of chick embryo cells (Cooper, 1955). Tissue culture fluid frequently contained more than 10^6 p.f.u./ml. Physical studies of this virus facilitated by the plaque count technique showed that it was inhomogeneous. Plaques of two sizes were always seen. Infective particles of at least three sizes were detectable by centrifugation, but the finding that one infective particle has a sedimentation constant of 20 S or less is of some interest. The slow sedimentation of this component may be due to a high degree of asymmetry (a filamentous particle perhaps?) or to a diminished particle density owing to the presence of lipid. The particle of 72 S corresponding to 19 m μ may be compared with the virus of foot and mouth disease which has a similar sedimentation constant (Bradish & Brooksby, 1960). We have not been able to relate the differences in plaque morphology with differences in sedimentation constants. If all the virus particles carried the same surface charge density irrespective of size, all should migrate together in an electric field. Electrophoresis of the virus, however, revealed the presence of at least two electrophoretic components. Whether these correspond with the two larger components separated by ultracentrifugation and which account for nearly all the infectivity, has not been determined. The behaviour of the virus

on electrophoresis was, however, quite different from that of any of the virus strains associated with bovine lumpy skin disease (Polson, unpublished). Inhibition of the infectivity of CBO virus by normal sera of several animal species has complicated serological studies, and investigation of this inhibitor is the subject of another paper (Turner *et al.* 1961). Although the virus is adsorbed by fowl and goose red cells, all attempts to demonstrate haemagglutination either directly or indirectly, were unsuccessful; nor was any evidence obtained of enzyme activity similar to that of the influenza viruses. The characters of the ECBO viruses are not yet sufficiently defined to be able to decide whether the CBO virus belongs to this group but some points of similarity make it a possibility.

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Neutralization of a Cytopathogenic Bovine Orphan Virus in Tissue Culture by Heat Stable Francis Type Inhibitors in Normal Animal Sera

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SUMMARY

A heat stable inhibitor present in the serum of normal animals was found to neutralize the infectivity of a cytopathogenic bovine orphan (CBO) virus. Its distribution and some physical, chemical and biological properties were examined. These properties suggest that the inhibitor for the virus infectivity is similar to, but not identical with Francis type inhibitors in normal animal sera.

INTRODUCTION

Many non-specific inhibitors of virus activity have been described in the tissues, body fluids or sera of normal animals. These inhibitors have been differentiated principally by the viruses which they affect, by their sensitivity to heat, enzymes or chemical reagents and by their ability to inhibit haemagglutination or to neutralize infectivity. Only a few have been obtained in pure or almost pure state. During an investigation of the properties of the Van den Ende strain of a cytopathogenic bovine orphan (CBO) virus (Kipps, Turner & Polson, 1961), it was noticed that the sera of normal animals of several species caused striking inactivation of the virus, shown by a decrease of the infectivity in chick embryos or the plaque-forming capacity in chick embryo tissue cultures. Further study revealed that the inactivating agent was relatively resistant to heat and resembled in many respects the heat stable serum components inhibiting haemagglutination by myxoviruses collectively known as the Francis, or α , inhibitor. The present paper describes some of the properties of the CBO virus inhibitor and its relationship with other known inhibitors.

METHODS

The viruses, the preparation of antisera, the propagation and titration of this CBO virus and the methods of neutralization are described in the preceding paper (Kipps *et al.* 1961).

Normal sera. Sera were obtained from clotted blood and heated to 56° for 30 min. to inactivate heat labile inhibitors. When serum samples were not used immediately they were stored at -20°.

Mucoproteins. Samples of meconium and of urinary and sheep salivary mucoprotein were obtained from the Walter and Eliza Hall Institute, Melbourne,

Australia. Egg white inhibitor was prepared according to the method of Sugihara, McDonell, Knight & Feeney (1955). Ovomucin from allantoic fluid was concentrated by dialysis of the fluid against pH 4.3 buffer at 4°. The resultant precipitate was redissolved in a minimal volume of 0.066 M-phosphate buffer (pH 8.2).

Haemagglutination inhibition (HI). Inhibition of haemagglutination was estimated by standard methods using 4 agglutinating doses (AD) of heated influenza B virus (Lee) as indicator.

Electrophoresis. Two methods were used for the separation of inhibitor in normal rabbit sera. One made use of the method of Polson (1952) allowing the simultaneous analysis of two solutions, and the other the technique of Svensson & Valmet (1955) in a vertical column of modified cellulose (Peterson & Sober, 1956).

Preparation of materials. Crystalline trypsin (Armour) was dissolved in phosphate buffer (pH 8.2) immediately before use. Commercial receptor-destroying enzyme (RDE, Phillips Roxane) was reconstituted according to the maker's instructions. Solutions of potassium periodate of various molarities were freshly prepared in distilled water. Zymosan and properdin-free sera were prepared by the method of Pillemer *et al.* (1954).

Buffer solutions. Dilute acetate buffer (pH 5.2) contained 2 g. NaOH and 4 ml. glacial acetic acid/l. Phosphate buffers were prepared by mixing 0.066 M- Na_2HPO_4 and 0.066 M- KH_2PO_4 to give the required pH value; pH 4.3 buffer contained 2.5 g. citric acid and 2.5 g. Na_2HPO_4 /l.

RESULTS

Neutralization of CBO virus by normal sera

The apparent neutralizing titre of the serum depends on the virus concentration (Table 1), but with 60–100 plaque-forming units (p.f.u.) most rabbit sera diluted 10^{-3} caused a 50% reduction in the plaque count. For this reason normal rabbit

Table 1. *Neutralization of CBO virus by normal rabbit serum*

Virus dilution	Serum dilutions				Control without serum
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	
10^{-2}	65	c	c	c	c
10^{-3}	15	sc	sc	c	c
10^{-4}	3	42	85	sc	sc
10^{-5}	1	11	34	60	60
10^{-6}	0	0	5	12	13

Numbers = average plaque count from duplicate plates; c, confluent plaques; sc, semi-confluent plaques.

serum was used for most of the experiments. Normal rabbit serum was also tested for its neutralizing activity in chick embryos, mice or monkey kidney tissue cultures against the viruses of influenza (PR 8), Newcastle disease, poliomyelitis (Mahoney), rabies (Flury), Rift Valley fever (pantropic and neurotropic), African horse sickness, yellow fever, West Nile and Semliki Forest viruses. In no instance was significant inhibition of infectivity demonstrated. The concentration of inhibitor in the heated sera of a variety of normal animals was determined against 60 p.f.u. of CBO virus. In order to allow as far as possible for individual variations, pooled sera from a

group of each species were used. The neutralizing effect of human, rabbit and guinea-pig sera was high, that of rat, bovine, horse and ferret sera somewhat lower, while fowl and mouse sera had little if any activity (Fig. 1).

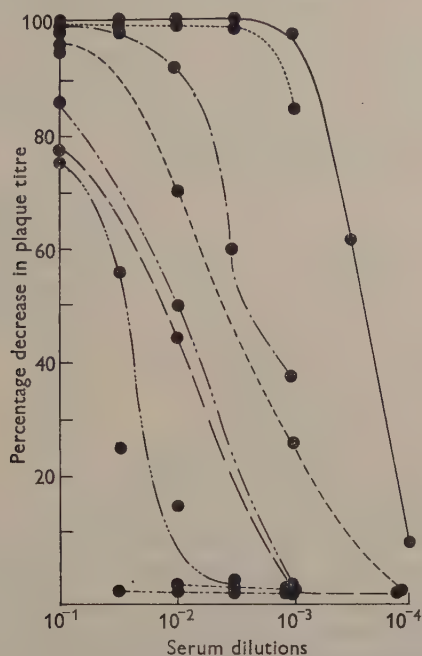


Fig. 1. The inhibitory effect of various normal animal sera against 60 p.f.u. of virus. Sera: ●—●, rabbit; ●...●, human; ●—...—●, guinea-pig; ●---●, rat; ●- - - -●, bovine; ●—●, horse; ●- - - -●, ferret; ●- - - -●, mouse; ●- - - -●, fowl.

Relation between inactivation of CBO virus and haemagglutination inhibition

The neutralizing powers of the pooled sera against CBO virus were compared with their potency as inhibitors of haemagglutination by heated influenza B virus (Lee) (Table 2). Although there was some parallelism between the neutralizing and HI

Table 2. *Haemagglutination-inhibition and CBO virus neutralization by normal animal sera*

Normal* animal sera	Reciprocal of dilution of serum causing inhibition of haemagglutination by 4 AD Lee B indicator virus	Reciprocal of dilution of serum causing 50 % reduction of plaque count with 60 p.f.u. CBO virus
Mouse	10	< 10
Ferret	2580	10-100
Fowl	80	< 10
Guinea-pig	320	100-1000
Human	1280	> 1000
Horse	5	100-1000
Bovine	5	100
Rabbit	80	> 1000

* Heated at 56° for 30 min.

powers of the different sera (in the cases of human and mouse sera for example), ferret serum was relatively more effective as an inhibitor of haemagglutination and rabbit serum more effective as a neutralizer of CBO virus. Mucoproteins from sources other than serum, though highly active as inhibitors of haemagglutination, failed to neutralize CBO virus (Table 3).

Table 3. *Haemagglutination-inhibition and CBO virus neutralization by mucoproteins*

Mucoprotein from	Concentration	Dilution causing inhibition of haemagglutination by 4 AD Lee B indicator virus	Inhibition of 60 p.f.u. CBO virus by undiluted mucoprotein
Meconium	10 mg./ml.	10^{-4}	nil
Sheep salivary mucin	1 mg./ml.	10^{-6}	nil
Urinary mucin	1 mg./ml.	10^{-4}	nil
Egg white in saline	20 % (v/v)*	$10^{-1.8}$	nil
Allantoic fluid	20 × conc.*	$10^{-1.8}$	nil

* See text, p. 416.

Concentration of the CBO inhibitor from rabbit serum

The precipitates formed by bringing the normal serum to half saturation with ammonium sulphate contained much of the virus neutralizing activity. Dialysis overnight against acetate buffer pH 5.2 was as effective. Little activity remained in the supernatant fluid and most of it could be recovered from the precipitate by extraction with phosphate buffer of pH 8.4 (Table 4). The sera of fowls which had been immunized against CBO virus contained neutralizing antibody which, however, was not precipitated at pH 5.2, the activity remaining in the supernatant fluid (Table 5). The agent was not sedimented by centrifugation of the serum at 33,000 rev./min. for 3 hr. in the no. 40 rotor of a Spinco Model L centrifuge, nor was it present in the lipid layer which separated during this treatment. When the serum was shaken with ether and the mixture centrifuged the agent remained in the aqueous phase.

Table 4. *Plaque count in presence of serum fractions diluted 1/10*

Sample	Plaque count
(1)* Globulins	2.0×10^3
(2) Albumin	1.1×10^6
(3) Virus alone	4.8×10^6
(4) pH 5.2 ppt	2.0×10^4
(5) pH 5.2 SNF	5.0×10^7
(6) Virus alone	4.0×10^7

* (1) Precipitate at half saturation with $(\text{NH}_4)_2\text{SO}_4$ redissolved in distilled water and dialysed against saline.

(2) Supernatant fluid from (1) dialysed to remove $(\text{NH}_4)_2\text{SO}_4$.

(4) Serum dialysed overnight at 4° against a large volume of dilute acetate buffer (pH 5.2). The precipitate redissolved in phosphate buffer (pH 8.4) dialysed against buffered saline and brought to the volume of the original serum.

(5) The supernatant fluid (SNF) from (4) dialysed against buffered saline.

Table 5. *Fractionation of CBO neutralizing agent in fowl immune serum by precipitation at pH 5.2*

The sera (normal or immune) were dialysed at 4° overnight against acetate buffer (pH 5.2). The precipitates were redissolved in phosphate buffer (pH 8.4). The supernatant fluids and the redissolved precipitates were dialysed against buffered saline and, after correction of their volumes to those of the original sera, were titrated against CBO virus in agar suspension cultures of chick embryo cells.

	Dilution of sample causing 50 % decrease in plaque count		
	Original serum	pH 5.2 precipitate	pH 5.2 supernatant fluid
Normal fowl serum	10^{-1}	N.D.	N.D.
Immune fowl serum	$10^{-3.8}$	$10^{-1.6}$	$10^{-3.5}$

N.D. = not done.

Electrophoresis of the CBO inhibitor

Normal rabbit serum which had been heated at 56° for 30 min. was submitted to electrophoresis in phosphate buffer (pH 8.2, ionic strength 0.1). The apparatus (Polson, 1952) allowed simultaneous analysis of two solutions and convenient sampling after electrophoresis. The progress of separation (descending boundaries only) was observed by the Lamm scale method. After electrophoresis at 4.5 V./cm. and 1.5° for about 4 hr., successive layers were removed (Polson, Joubert & Haig, 1946), serially diluted and tested for neutralizing activity against CBO virus on agar suspension cultures of chick embryo cells (Cooper, 1955). Photographs of the scale taken before sampling and after removal of each fraction enabled the electrophoresis diagram and the relation thereto of the samples to be accurately determined. The electrophoresis diagram of the serum (Fig. 2) shows the position of the samples tested and relative neutralizing power. Most of the inhibitor migrated to a position between the albumin and α globulin.

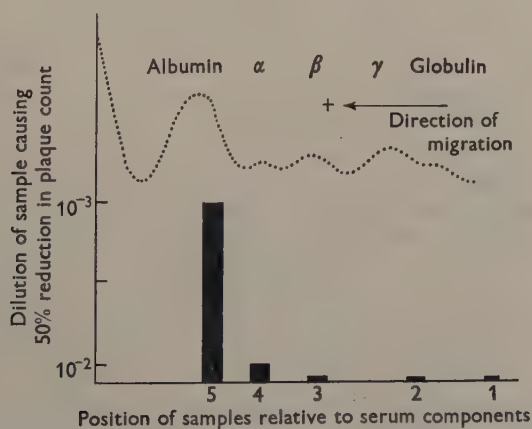


Fig. 2. Electrophoresis of normal rabbit serum at pH 8.2, 4.5 V./cm. and 1.5° for 4 hr. showing the position of the virus inhibitor.

Partially purified CBO inhibitor was also submitted to zone electrophoresis at pH 8.6. The fraction of normal rabbit serum precipitated at pH 5.2 was redissolved in phosphate buffer (pH 8.6) and centrifuged at 12,000 rev./min. for 15 min. The sediment and a small lipid layer which collected at the surface were discarded. The solution, which was faintly blue, was dialysed against borate buffer (pH 8.6) and submitted to electrophoresis at 46–47 mA. for 19 hr. in a vertical column of modified cellulose (Peterson & Sober, 1956) in the apparatus of Svensson & Valmet (1955) adapted to permit continuous recirculation of buffer through the electrode vessels. Buffer streams leaving the anode and cathode compartments were mixed and returned to an overhead supply vessel by a small pump. The current through the apparatus and the pK value of the buffer then remained constant throughout a run. After electrophoresis the column contents were displaced by buffer and collected fractionally. The fractions were tested for ultraviolet absorption at 275 m μ , neutralizing activity against CBO virus and haemagglutination inhibition against heated Lee B virus (Fig. 3).

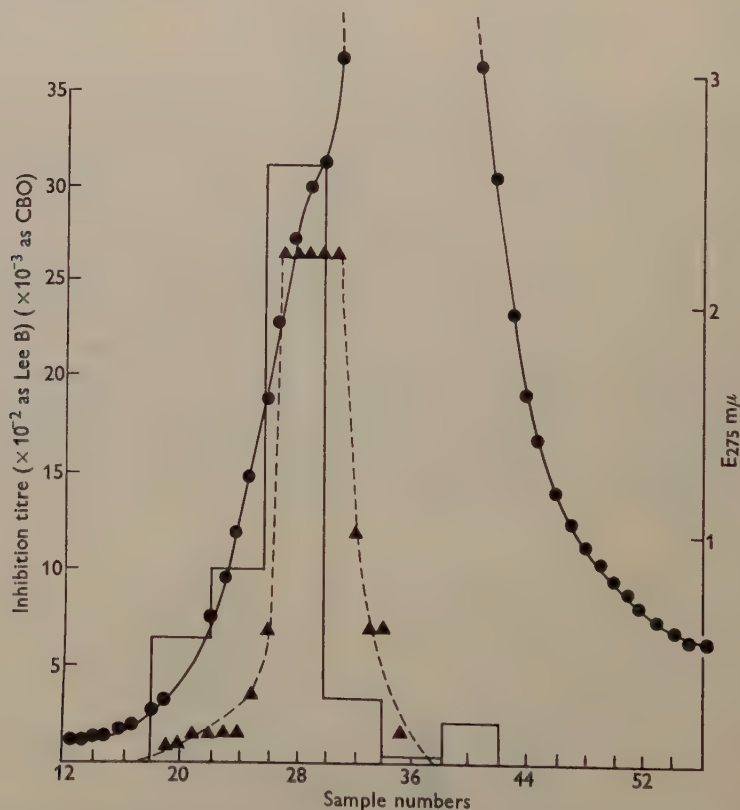


Fig. 3. Column electrophoresis of partially purified inhibitor. ●—●, Light absorption; ▲—▲, inhibition of 4 AD Lee B indicator virus; □, 50% inhibition of CBO virus.

The effect of heat on the CBO inhibitor in normal rabbit and human sera

The neutralizing power of normal rabbit serum against CBO virus was not significantly decreased by heating for 1 hr. at 56°. Portions of normal rabbit serum diluted

1/5 in saline were heated in a water-bath for 30 min. at various temperatures above 56° and assayed for haemagglutination inhibition (HI) of Lee B virus and neutralization of CBO virus. In the temperature range 65°–100° the neutralizing activity was more sensitive to heat than the HI activity; both were, however, still detectable in the sample heated at 100° for 30 min. (Fig. 4).

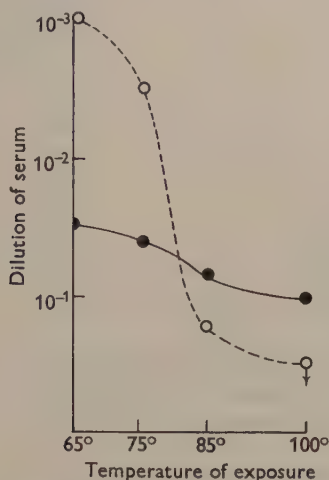


Fig. 4. The effect of heat on the inhibitors in normal rabbit serum to CBO virus and influenza (Lee B) virus. ○---○, Serum dilutions causing 50% decrease in plaque count of CBO virus; ●—●, serum dilution causing inhibition of 4 AD of influenza virus (Lee B).

Zhdanov, Hamburg & Svet-Moldavsky (1959) showed that the HI activity of normal human serum towards certain strains of Type A₂ influenza virus was greatly increased by heating at 85°. A sample of normal human serum heated at this temperature for 15 min. was found to have lost most of its neutralizing activity against CBO virus whereas its HI titre when tested against influenza virus A₂/Cape Town/59 had increased from 60 to more than 2560.

The effect of receptor-destroying enzyme (RDE) and active influenza B virus (Lee) on the CBO inhibitor in normal rabbit serum

One ml. portions of normal rabbit serum were treated either with 5 ml. RDE solution or 5 ml. saline, incubated for 18 hr. at 37°, heated for 1 hr. at 56° and assayed for neutralization of CBO virus and HI of influenza B indicator. Similarly, 1 ml. amounts of normal rabbit serum were treated with 1 ml. volumes of active influenza B virus (Lee) purified according to Burnet (1948) and containing 400 AD. The mixtures were incubated at 37° for 18 hr., heated to 65° for 30 min. and diluted for assay. Controls were prepared in the same way except that active Lee B virus was replaced by heat inactivated influenza B virus or by tap water. The CBO inhibitor resembled the haemagglutination inhibitor in normal rabbit serum in its sensitivity to the enzymes in RDE and active influenza B virus (Table 6).

The effect of trypsin on the CBO inhibitor in normal rabbit serum

One ml. portions of normal rabbit serum were treated either with 1 ml. of buffer (pH 8.2) containing 8.0 mg. crystalline trypsin or 1 ml. buffer without trypsin. The mixture was incubated at 37° for 20 hr., treated with 3 ml. saline containing 12 mg.

Table 6. *The effect of RDE and active Lee B influenza virus on the inhibitor in normal rabbit serum*

For details see text.

Normal rabbit serum treated with	% decrease of inhibitory activity of samples of serum against	
	CBO virus	Heated Lee B virus
(1) Nil	0	0
(2) Saline	2	5
(3) RDE	85	94
(4) Heated Lee B virus	4	25
(5) Water	4	25
(6) Active Lee B virus	85	94

soya-bean trypsin inhibitor, and assayed for CBO virus neutralizing activity and HI of influenza B (Lee) indicator virus. The enzyme caused considerable reduction in both types of inhibitory activity (Fig. 5).

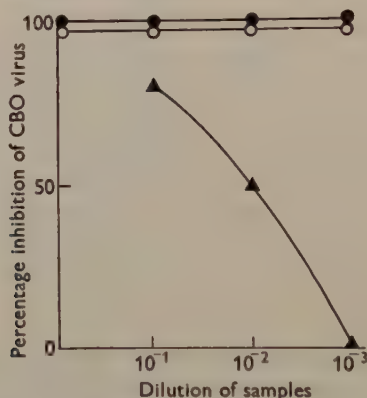


Fig. 5. Effect of trypsin on the inhibitor to CBO virus in normal rabbit serum. ○—○, control samples without trypsin (HI titre with heated Lee B virus = 160). ▲—▲, trypsin-treated sample (HI titre with heated Lee B virus < 5).

The effect of potassium periodate

Portions of normal rabbit serum were mixed with equal volumes of either saline or of one of a series of dilutions of a freshly prepared solution of potassium periodate. The mixtures were held at 37° for 1 hr., treated with glycerol (0.02 ml.) to destroy excess reagent and assayed for their effect on CBO and Lee B viruses. Both types of activity were about equally sensitive to periodate treatment (Fig. 6).

Differentiation of the CBO inhibitor from properdin

Treatment of freshly drawn normal rabbit serum with a concentration of zymosan which had proved sufficient to remove the C3 component of complement in normal rabbit serum had no effect upon its neutralizing activity against CBO virus. Normal

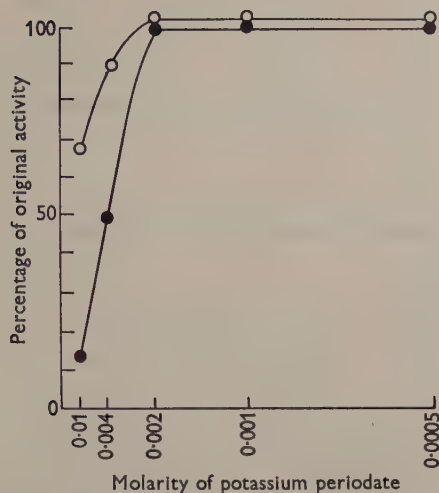


Fig. 6. Effect of periodate on CBO and Lee B inhibitors in normal rabbit serum.
○—○, against heated Lee B virus; ●—●, against CBO virus.

human serum containing 5.7 units properdin/ml. and a sample of RP (Pillemer *et al.* 1954) prepared from it by removing all detectable properdin with zymosan, gave identical results in the neutralization test against CBO virus.

The effect of RDE and active influenza B virus (Lee) on the virus+inhibitor complex

During the experiments recorded above it was noticed that RDE and active influenza B virus (Lee) were capable of liberating active CBO virus from the inactive complex which it forms with the inhibitory agent. A mixture of undiluted normal rabbit serum and suitably diluted CBO virus was allowed to stand at room temperature for 1 hr. and divided into six portions. To one portion was added 1 ml. of active influenza B virus (Lee), to the second heat-inactivated influenza B virus (Lee) (56° for 30 min.), to the third 5 ml. of active RDE and to the fourth 5 ml. of heat-inactivated RDE (100° for 15 min.). Hanks solution (5 ml.) was added to portions 5 and 6. Portions 1–5 were incubated overnight at 37° and portion 6 was held at 4°. Two control mixtures of CBO virus and Hanks solution were similarly allowed to stand at room temperature for 1 hr. They were then diluted with 5 ml. Hanks solution and one was incubated overnight at 37° and one held at 4°. On the following morning RDE, where present, was inactivated by the addition of sodium citrate, and all the solutions were diluted to 10 ml. and assayed for HI activity with heated influenza B virus (Lee) and for active CBO virus. CBO virus was only slightly inactivated during incubation at 37° and sufficient inhibitor was supplied in the rabbit serum to inactivate the CBO virus completely. No reactivation of virus occurred as a result of the incubation or dilution, but active CBO virus was released from combination with the neutralizing agent of rabbit serum by RDE and active influenza B virus (Lee), both of which destroyed the HI activity of the serum against

heated influenza B virus (Lee) (Table 7). The recovery of active virus was about 50 % when RDE was used, about 10 % with active influenza B virus (Lee).

Table 7. *The effect of RDE and active influenza B virus (Lee) on the CBO virus + inhibitor complex*

CBO virus + inhibitor complex treated with	Titre of CBO virus p.f.u./ml.	Inhibitor content against 4 AD of Lee B indicator virus
(1) Active Lee B*	1.3×10^3	< 10
(2) Heated Lee B	Nil	40
(3) RDE	6.7×10^3	< 10
(4) Heated RDE	Nil	40
(5) Hanks at 4°	Nil	40
(6) Hanks at 37°	Nil	40
(7) CBO virus control at 4°	34×10^3	—
(8) CBO virus control at 37°	12.5×10^3	—

* Active Lee B did not produce plaques on agar suspension cultures of chick embryo cells.

DISCUSSION

The infectivity of the Van den Ende strain of CBO virus, demonstrable by the formation of plaques in chick embryo cell monolayer cultures, cytopathic effect in chick tissue cultures or mortality in chick embryos, appeared to be almost completely neutralized by normal rabbit sera heated to destroy inhibitors of the heat labile type. Such sera had no effect upon the infectivity of all the other viruses tested in this investigation with the exception of one strain of enteric cytopathogenic bovine orphan virus (ECBO).

In the normal sera examined, the species distribution of the CBO virus inhibitor differed considerably from that of the Francis type of inhibitor of influenza B (Lee) indicator virus. This difference was most marked in rabbit and ferret serum. This lack of parallelism in inhibitory activity has been demonstrated by Ananthanarayan & Paniker (1960) who showed that among the myxoviruses considerable variations occurred in the susceptibilities of different strains to the same or similar inhibitors. Conversely, it may be true that haemagglutination inhibition is not due to one substance but to a number of serum mucoproteins of varied activity towards different strains of virus.

The electrophoretic behaviour of the α inhibitor in animal sera was examined by Tyrrell (1954), Harboe, Raenaas & Oppedal (1958) and Levy, Norman & Wagner (1959) and when allowance is made for different techniques, their results indicate that the active substances have mobilities approximating to that of the α globulin fraction. The CBO inhibitor in normal rabbit serum has a similar mobility. The peaks of inhibitory activity for haemagglutination by Lee B virus and for the infectivity of CBO virus were coincident.

Confirmation of the association of the inhibitor with the globulins was obtained when sera were half saturated with ammonium sulphate. It is not clear whether the precipitation of the inhibitor at pH 5.2 is a specific property of the substance or whether it was due to adsorption to other serum constituents precipitated at this

pH value. It provided, however, a further method of eliminating albumin and distinguishing it from immune γ globulin. In addition, the inhibitory activity was shown to be independent of properdin.

The heat stability and the sensitivity of the inhibitor to periodate and trypsin suggest that it is a mucoprotein and the presence of sialic acid may be inferred from its sensitivity to neuraminidase. Mucoproteins from other sources having high haemagglutination inhibition activity for the influenza B (Lee) indicator virus were without effect upon CBO virus infectivity, suggesting that CBO virus is more restricted than some of the influenza viruses in the range of mucoproteins with which it can combine.

The experiments with the virus+inhibitor complex show that, in contrast to active influenza virus, the complex is not dissociable by time or temperature, nor is it dissociated on simple dilution. The adsorption of CBO virus to certain red cells which are not agglutinated (Kipps *et al.* 1961), and to the inhibitory substance in normal animal sera, can be reversed by RDE and active influenza B virus (Lee), implying that while the CBO virus combines with inhibitor in a manner similar to the influenza viruses it lacks the necessary enzyme for its own elution.

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Titration of Trachoma Virus with Observations on Yolk-sac Infection and Sensitivity to Oxytetracycline, using the Single-dilution method

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SUMMARY

The T'ang strain of trachoma virus was titrated in chick embryo yolk sac by a single-dilution method (Golub, 1948), using 3-6 eggs/dilution. The limitations incurred by the use of small numbers of eggs are discussed. The method was used to make observations on the fate of virus after inoculation into the yolk sac, and to study the sensitivity of virus to oxytetracycline. The method was found to be unsuitable for titration of neutralizing antibodies in fowl serum.

INTRODUCTION

The experiments described in this paper were carried out for two reasons. Firstly to examine the efficacy of Golub's (1948) single-dilution method of titration when applied to the T'ang strain (T'ang, Chang, Huang & Wang, 1957) of trachoma virus, using smaller numbers of fertile eggs than Golub employed for psittacosis virus; secondly, to use this titration method to make some observations on the behaviour of the virus in the chick embryo and yolk sac.

METHODS

Virus. The T'ang strain of virus was used throughout. It was received as twenty-sixth yolk-sac passage material from Dr L. H. Collier (M.R.C. Trachoma Research Unit, Lister Institute, London). Virus stocks were prepared by homogenization of infected yolk sacs made up to a total volume of 10 ml. with sucrose+potassium glutamate (SPG) solution (Bovarnick, Miller & Snyder, 1950). The crude homogenate was distributed in 0.5 or 1.0 ml. amounts in ampoules, rapidly frozen, and stored at -70° . Fresh stocks were made about every 2 months.

Inoculation of yolk sacs. Except when stated otherwise, virus was injected in 0.2 ml. quantities into the yolk sacs of eggs after 7 days' incubation at 37° . The eggs were then incubated at 35° and candled daily. Embryos dying within 3 days of inoculation were discarded. After the first deaths had occurred eggs were candled twice daily. Smears were made of yolk sacs of all dead embryos and stained with 1/5 (v/v) Giemsa stain (G. T. Gurr, London) in pH 6.8 buffer for 30 min. From more than a hundred such smears made in the early stages all but a few contained elementary bodies; therefore embryos found dead with negative smears were not included in the results. All dilutions of virus were made in sucrose+potassium glutamate solution.

Oxytetracycline. Soluble tablets of oxytetracycline hydrochloride (Pfizer) were used to prepare fresh solutions within 1 hr. of injection into eggs. The tablets were dissolved in phosphate buffered saline (pH 7·2). Sensitivity determinations were made by inoculating into 7-day yolk sacs 0·2 ml. of a mixture of stock virus and oxytetracycline solution in which the final concentration of virus was 1/10 and the amount of oxytetracycline was as shown (Tables 5 and 7).

Antiserum. Antiserum was prepared by injecting 0·5 ml. of stock virus intraperitoneally into a hen at weekly intervals for 5 weeks. Blood was collected by heart puncture before the first injection and 1 week after the last injection. The second serum gave a titre of 1/128 in an indirect complement-fixation test against a suspension of virus partly purified by extraction with ether followed by differential centrifugation.

Neutralization test. Virus and fowl antiserum were mixed to give the final dilutions shown in Table 8. After incubation at 35° for 15 min. 0·2 ml. of each mixture was inoculated into three 7-day-old eggs which were incubated at 35° and candled daily thereafter.

RESULTS

Assessment of the single-dilution method of titration

Golub (1948) described a single-dilution method of titrating the infectivity of psittacosis virus, using 10–30 fertile eggs per dilution, and estimating the mean survival time after inoculation of virus. It was found possible to titrate the T'ang strain by this method, with fewer eggs than Golub used, and a consequently diminished accuracy. For some purposes, however, 3–4 eggs per point enable limited conclusions to be drawn from the mean survival times. The regression of survival times on \log_{10} LD₅₀ inoculated was examined by inoculating 4–6 eggs with tenfold dilutions of stock virus. Eggs were incubated for a further 14 days, and the LD₅₀ calculated by Thomson's Method of Moving Averages (Thomson, 1947). Embryos surviving for the whole 14 days were considered to be uninfected. The mean values of the survival times, even for such small groups of eggs, lay fairly close to a straight line drawn by eye (Fig. 1), which supported the idea that the relationship of survival time to infectivity of the inoculum was the same as that described by Golub for psittacosis virus.

An estimate of the number of elementary bodies in one LD₅₀ was made by spreading 0·02 ml. of stock virus evenly over a ruled area of 4 cm.² on a slide, staining with Giemsa, and counting the number of bodies in five microscope fields at a magnification of 1700. Two values for the LD₅₀ obtained in this way were 7600 and 554 elementary bodies. Several similar counts were performed during these experiments on yolk sac homogenates prepared from embryos dying between the fifth and tenth days after infection. These gave values of 10^9 to 10^{10} elementary bodies per yolk sac at death. The multiplication, in terms of elementary bodies, of an inoculum which killed embryos 14 days after infection was therefore of the order of 10^6 to 10^7 times.

An assessment of the sensitivity of the method is given in Table 1, taking the regression of survival time on dilution of virus inoculated as linear. A difference in infectivities equivalent to a dilution of less than about one in thirty would not be expected to show as a significant difference between mean survival times.

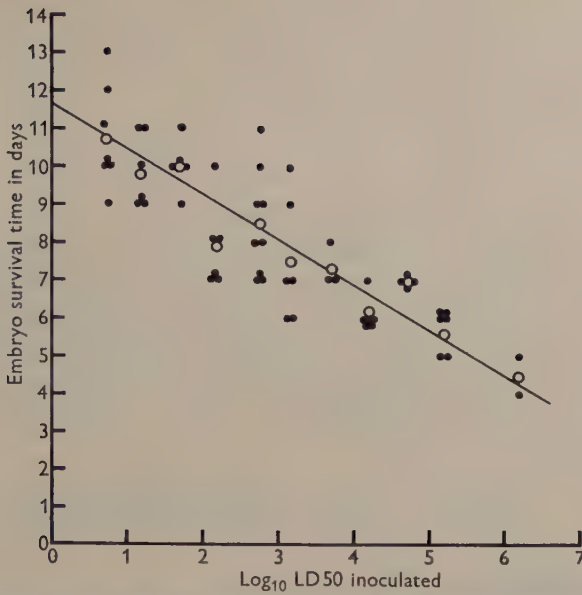


Fig. 1. Regression of survival time of embryos on \log_{10} LD 50 of *Trachoma virus* (T'ang strain) inoculated into the yolk sac. O, mean survival times.

This application of the single-dilution method of Golub is therefore extremely coarse, but it is economical and will enable useful conclusions to be drawn provided that the limitations are borne in mind. To avoid the additional sources of variation involved in determining the LD 50, the results of titrations are expressed throughout this paper as the mean survival time (MST) of a group of 4–6 embryos after injection

Table 1. *Sensitivity of method of titration*

\log_{10} dilution of virus inoculated	Survival times of embryos (days)	
	1	2
0	4, 5	7, 7, 7, 7
1	5, 5, 6, 6, 6	7, 7, 8
2	6, 6, 6, 6, 6, 7	7, 7, 7, 9, 10
3	6, 6, 7, 7, 9, 10	7, 7, 7, 9, 10
4	7, 7, 7, 8, 8, 10	10, 10, 11, 13
5	9, 9, 9, 10, 11, 11	—

If Mean Survival Time (MST) = $K \times \log_{10}$ dilution of inoculum, 1 day = about 1 \log_{10} unit (Fig. 1). Variance of single observation = about 1 day. Variance of MST of 4 embryos = about $\frac{1}{4}$ day. Variance of difference between MST values in groups of 4 embryos = $\frac{1}{2}$ day, and s.d. = $1/\sqrt{2}$ day. Therefore difference will be significant at 95 % level if it exceeds $2 \times$ s.d. = about $\sqrt{2}$ = about 1.4 days.

on the seventh day of incubation of 0.2 ml. of virus suspension. The abbreviations in brackets after the abbreviation MST indicates the source of the material inoculated, for example, MST(YS), means that the figure given is the mean survival time of a group of eggs after the injection of yolk-sac suspension; in the text this may be expressed as the MST of a suspension. Embryos surviving the whole

14-day period are treated for quantitative purposes as though they died on the fourteenth, so that an MST of 14 days implies that all the embryos survived. This is necessary to deal with such a result as the death of one embryo in a group before 14 days, and the survival of the others.

Observations on growth of the virus

Effect of infection on embryo and yolk-sac development

A possible explanation for the relationship between survival time and inoculum is that the growth rate is independent of the size of the inoculum and that the embryo will die when a certain number of cells has been irreparably damaged. There could also be a slow toxic action of the material inoculated. The increase in weight of small numbers of infected embryos was not greatly different from that of uninfected embryos (Table 2). This is not the kind of result to be expected of some toxic effect beginning soon after inoculation.

Table 2. *Increase in weight of infected and uninfected embryos*

Days after injection of 0.2 ml. virus suspension* into 8-day eggs	Weights (g.) of infected embryos	Weights (g.) of uninfected embryos
0	—	0.7, 0.7, 0.9, 0.9, 1.2
1	1.3, 1.5, 1.8	1.3, 1.4, 1.5
2	1.4, 1.6, 1.6	1.8, 1.9, 2.0
3	2.5, 2.6, 2.6	2.0, 2.2, 2.6
5	4.5, 5.8	3.7, 4.9, 5.0
6	5.6, 6.0, 6.1	6.1, 7.0, 7.9

* Stock virus diluted 1/10. Survival time 6-7 days.

In another experiment yolk sacs harvested at intervals after infection increased in weight in the same way as uninfected yolk sacs until a day or two before death of the embryo, at which time they actually fell in weight (Fig. 2).

Distribution of virus after inoculation of yolk sac

The infectivity of yolk after inoculation with virus was determined by removing the shell over the air-space and aspirating a quantity of yolk from the yolk sac which was easily visible beneath the shell membrane. The aspired yolk was used to inoculate other eggs in 0.2 ml. amounts, without further dilution (Table 3). Suspensions for the titration of yolk-sac infectivity were prepared by washing the sac carefully in two changes of phosphate buffered saline and homogenizing it in a total volume of 10 ml. of sucrose + potassium glutamate solution. When the homogenate was not to be inoculated into eggs at once it was rapidly frozen and stored at -70° until required. Storage of virus in this way caused some loss of infectivity (Table 3). 0.2 ml. of homogenate was inoculated into the eggs used for titration. In one experiment embryos were titrated for infectivity in the same way as yolk sacs (Table 3). When the volume of yolk is taken as 20 ml., the dilution of the inoculum after injection is 1/100, so that the infectivity of yolk immediately after inoculation

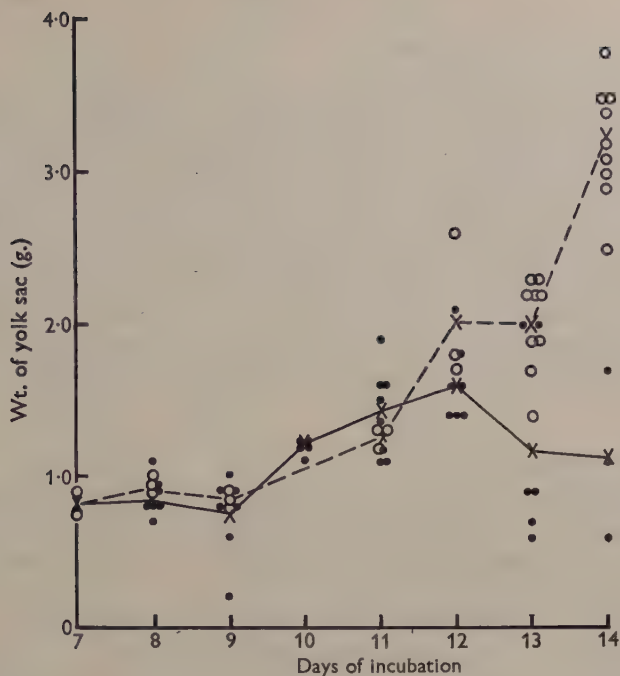


Fig. 2. Growth of normal yolk sacs and yolk sacs infected on the seventh day of incubation.
 O, Normal yolk sacs; ●, infected yolk sacs; ×, mean weights.

would be expected to show an MST 2-3 days greater than the inoculum and this expectation is borne out by the figures (Table 3). One day after injection infectivity could still be recovered from the yolk in quantities similar to that present immedi-

Table 3. *Infectivity of yolk, yolk sacs, and embryos at intervals after inoculation of virus*

Expt. no.	MST of inoculum	Infectivity of	Days of incubation at 35° after inoculation						
			0	1	2	3	4	5	6
28b	6.4	Yolk*	—	14	—	—	—	—	—
		Yolk sac*	—	14	9.0	7.0	7.0	5.0	5.0
		Embryos*	—	14	14	11.0	—	6.3	8.0
56	5.4	Yolk sac*	—	13.5	7.5	—	6.0	6.8	—
82a	6.0	Yolk	8.0	7.0	—	—	—	—	—
		Yolk sac	—	9.5	—	—	—	—	—
82b	6.0	Yolk	7.0	7.0	—	—	—	—	—
		Yolk sac	—	11.0	—	—	—	—	—
78	9.0	Yolk	11.0	10.0	—	—	—	—	—
		Yolk sac	—	10.0	—	—	—	—	—

* Stored at -70° for 1 week before titration.

ately after injection. Infective virus could also be recovered from washed homogenized yolk sacs 1 day after inoculation. The presence of significant amounts of infective virus in the yolk even 1 day after inoculation implies that absorption of virus by entodermal cells may continue for too long after injection to make this system a useful one for studies on the kinetics of virus growth.

Phagocytosis of Indian ink by yolk sac

In order to test the possibility of dispersal of virus throughout the whole egg contents after inoculation a 10 % (v/v) suspension of 'Pelikan' ink was injected into the yolk sacs of several 7-day-old eggs which were incubated at 35°. The contents of these eggs were examined at intervals after injection. Fifteen minutes after injection washed yolk sacs showed a faint grey band (Pl. 1, fig. 1). Over the next 24 hr. this band increased in density and slightly in width, and at this time ink was present only in the yolk-sac contents. Histological sections showed widespread uptake of ink by entodermal cells with a heavy concentration in the position of the band. There may therefore not be much dissemination of inoculated virus throughout the egg, and phagocytosis may play at least some part in the entry of virus into cells.

Effect of oxytetracycline on infection

The embryos were protected by 20 µg. of oxytetracycline against all the doses of virus used. Below this amount of oxytetracycline the sensitivity was inversely related to the dose of virus (Table 4). For example, 5 µg. oxytetracycline failed to

Table 4. *Effect of size of inoculum of virus on sensitivity to oxytetracycline (OT)*

MST of inoculum (days)	...	5.8	7.2	8.8
MST after	{	20 µg.	14	14
OT (days)		10 µg.	11.8	—
		5 µg.	6.8	14
		2.5 µg.	5.8	9.3
				14
				11.3

protect against an inoculum whose MST was 5.8 days, whereas the same dose protected against an inoculum whose MST was 7.2 days.

The phenomenon of delayed death shown in the first column of Table 4 was observed several times in this system, and a similar effect was reported for this and other antibiotics acting on the psittacosis-lymphogranuloma group by Katz (1956) and Jawetz & Hanna (1960*b*). It must be due to one of three causes, decay of antibiotic, retardation of virus growth, or appearance of a resistant variant. The third possibility was examined by testing the sensitivity of the virus recovered from the yolk sacs in the eggs which showed a delayed death after 10 µg. oxytetracycline. No increase of resistance was detected.

Table 5. *Effect of time of addition of oxytetracycline (OT) on its ability to protect infected embryos*

Days after infection on which 50 µg. OT added	0	1	2	3	4	5	No OT
MST	14	14	14	13.4	10.0	8.2	6.5

It was interesting to know at what time after inoculation death could no longer be prevented by antibiotic treatment. This method was used very successfully by Keppie, Smith & Harris-Smith (1955) in studying infection of guinea-pigs with *Bacillus anthracis*. After inoculation of virus, groups of 4–6 eggs were injected at

24 hr. intervals with 0.2 ml. oxytetracycline solution containing 50 μ g. antibiotic. Embryos were completely protected up to 2 days after infection, almost completely after 3 days, and with decreasing efficiency after 4 and 5 days (Table 5). Several determinations were made of the amount of infectivity that could be recovered from yolk sacs some days after the administration of a protective dose of oxytetracycline (Table 6). Infective virus could be recovered from the yolk sac 14 days after injection when oxytetracycline was injected more than 2 days after virus injection.

Table 6. *Survival of virus in yolk sac after oxytetracycline (OT)*

Days after injection on which OT given	Infectivity (MST) of inoculum	μ g. OT injected	Infectivity (MST) of infected yolk sacs at these intervals (days) after inoculation of virus						
			2	3	4	6	8	10	14
0	7.0	100	14	—	—	—	—	—	14
1	6.5	100	—	—	—	—	14	—	—
2	6.5	100	—	—	—	—	14	—	14
3	6.5	100	—	—	—	—	7.7	—	8.5
3	7.9	50	—	11.8	—	—	—	—	14
3	7.9	50	—	12.0	—	—	—	—	14
3	6.0	50	—	(7)*	—	10.0	9.0	9.3	(8)*
4	6.5	100	—	—	—	—	7.5	—	—
5	6.5	100	—	—	—	—	5.0	—	9.0

* Survival time of one embryo.

Table 7. *In vitro effect of oxytetracycline (OT)*

Virus 0.5 ml. + yolk 10 ml. + OT 1 mg. at 35°/24 hr. 0.2 ml. of 1:10 dilution (containing 2 μ g. OT + stock virus 1/200) injected into yolk sac.

Duration of exposure of virus to OT	MST of virus/OT mixture (days)
0 min.	10.0
24 hr.	10.6

There was some decrease in the degree of infectivity which could be due to thermal inactivation, metabolic activity of the cells, or mechanical removal of virus. A slow viricidal effect of oxytetracycline cannot strictly be excluded, but this is made unlikely by the absence of a viricidal effect in 24 hr. incubation of virus in the presence of 100 μ g. oxytetracycline/ml. (Table 7), a concentration many times greater than that to which intracellular virus would have been exposed. These experiments provided no evidence against the idea that oxytetracycline exerts a virustatic effect on this strain of trachoma virus.

Table 8. *Neutralization of T'ang virus (MST's of serum/virus mixtures)*

Final virus dilution	Figures in parentheses = % 'neutralization'.			Normal serum
	Final antiserum dilution			
	1/2	1/10	1/100	
10 ⁻¹	8.0 (99.3)	5.5	5.5	5.5
10 ⁻²	9.0 (99.7)	7.0	5.6	6.0
10 ⁻³	10.0 (97.8)	8.3	8.0	8.0

Neutralization of virus

Fowl antiserum diluted 1/2 produced a prolongation of mean survival time of 2-3 days with all three dilutions of virus. There was no prolongation with the higher dilutions of serum (Table 8).

DISCUSSION

Dependence of survival time upon the amount of trachoma virus inoculated into yolk sacs was shown by Jawetz & Hanna (1960*a*) who used three strains isolated in the United States. They found that 10^5 LD₅₀ doses killed embryos in 5-6 days, and 10 LD₅₀ killed in 10-12 days, which agrees with the survival times recorded here for the T'ang strain. Since appreciable amounts of infectious virus were found in embryos 3 days after infection (Table 3), and embryos could survive for 2 weeks after inoculation of virus when they were protected by the virustatic action of oxytetracycline injected 3 days after infection (Table 6), it is unlikely that the virus kills embryos by a toxic action of the kind described in mice by Bell, Snyder & Murray (1959). The loss of weight of infected yolk sacs (Fig. 2) may have been due to the fact that a high proportion of the weight of the yolk sac consists of materials in transit from yolk to embryo (Romanoff, 1960); it may be that when infection has damaged cells beyond a certain point they are no longer able to take up yolk material, but can still pass on, or metabolize, what they already contain. On this hypothesis lack of nutrients contributes to the death of the embryo. Such fatal damage to the entodermal cells must occur a short time before the death of the embryo, since infected embryos weighed the same as uninfected ones, and the growth curve of infected yolk sac followed that of uninfected until a day or two before death (Fig. 2).

Katz (1956) found that feline pneumonitis virus was inhibited by 31 μ g. tetracycline or oxytetracycline. Jawetz & Hanna (1960*b*) found that two of their strains were inhibited by 2 μ g. tetracycline/ml. (that is, 1.0 μ g. in the inoculum). The T'ang strain was inhibited completely by 20 μ g. oxytetracycline.

A possible explanation of the inverse relationship of sensitivity to virus dose below 20 μ g. oxytetracycline (Table 4) may be that an intracellular threshold concentration of oxytetracycline is attained after injection of less than 20 μ g. which can inhibit the development of one infective particle/cell, such as may occur after a small inoculum, but not of two or more, which would be present in many cells after a large inoculum. Katz (1956) found that the activity of tetracycline on feline pneumonitis virus was independent of the inoculum. His figures suggest that feline pneumonitis virus is more lethal for chick embryos than trachoma virus. For example 10^4 LD₅₀ doses of feline pneumonitis virus gave a mean survival time of 5.2 days, whereas the same dose of trachoma virus killed in 7.0 days. It is therefore possible that at a dosage level of 10^5 LD₅₀ doses of feline pneumonitis virus most infected cells contained one particle only. If the hypothesis is correct that an intracellular threshold amount of antibiotic is required to inhibit one intracellular particle, the difference between the findings of Katz and those in Table 4 can be explained.

The failure of a fowl antiserum to neutralize the T'ang strain completely is disappointing. Even the slight prolongation of survival time may have been due, for example, to agglutination rather than neutralization. The system itself is not very



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suitable for demonstrating neutralization, since a decrease of infectivity to less than one-thirtieth of the initial value must be attained before a significant difference can be shown in the mean survival times (Table 1). If the figures in Table 8 are taken at their face value, to indicate neutralization, and the MST values converted to approximate LD50 dose by using Fig. 1, the percentage neutralization is seen to be of the same order with all three dilutions of virus. This recalls the Percentage Law of Andrewes & Elford (1933).

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EXPLANATION OF PLATE 1

Fig. 1. 7-day yolk sac 1 hr. after injection of 'Pelikan' ink into the yolk. *a*, Band of heavy uptake of ink.

Influence of Certain Sterols and 2:4-Dinitrophenol on Phosphate Accumulation and Distribution in *Tetrahymena pyriformis*

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SUMMARY

Orthophosphate accumulation by *Tetrahymena pyriformis* W was enhanced by stigmasterol, β -sitosterol or cholesterol and depressed by 2:4-dinitrophenol (DNP) to an extent determined by the concentration of DNP and hydrogen ion in the suspension medium. The depression of phosphate accumulation by DNP was mitigated by the addition of stigmasterol, the degree of annulment being dependent in part on the stigmasterol concentration. The distribution of accumulated phosphate was not significantly altered by the addition of sterol and/or DNP under the conditions used, indicating that these compounds may influence entry of orthophosphate by affecting a membrane phenomenon.

INTRODUCTION

The interaction of sterols and 2:4-dinitrophenol (DNP), colchicine and several steroids in the growth of *Tetrahymena pyriformis* has been reported (Conner, 1957; Conner & Nakatani, 1958; Conner, 1958, 1959*a, b*). The growth inhibitors represent a wide variety of compounds that have been reported to interfere with phosphate metabolism. Colchicine (Lettre, 1951) was reported to inhibit ATPase activity; steroids have been shown to interfere with oxidative phosphorylation and to stimulate 'latent' ATPase activity in mitochondria (Wade & Jones, 1956*a, b*), as does 2:4-dinitrophenol (Loomis & Lipmann, 1948; Hunter, 1951). DNP is believed to lead to a loss of some of the energy which is normally available to cells through oxidative processes; accordingly, energy-dependent growth and maintenance become diminished. The lowering of phosphate accumulation in *Tetrahymena* in the presence of DNP was reported by Conner, Goldberg & Kornacker (1961). Van Wagtendonk & Wulzen (1951) showed that guinea-pigs fed a diet deficient in plant sterols ('anti-stiffness' factor) developed a syndrome most readily explained in terms of a disturbance of phosphate metabolism. These findings, coupled with the DNP inhibition of phosphate accumulation in *Tetrahymena*, led to a study of the relationship of sterols and DNP to phosphate accumulation and distribution in this ciliated protozoan.

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METHODS

The organism used was *Tetrahymena pyriformis* W; it was grown in a proteose peptone medium at pH 6.5 for the phosphate accumulation studies. For the distribution experiments the organism was grown in a modification of the chemically defined medium previously used for growth studies (Conner, 1959*b*). In the present study the phosphate concentration was decreased by a factor of ten and the Tween 80 was omitted.

Stigmasterol was added to the suspension medium as an aqueous emulsion, prepared by dissolving the sterol in absolute ethanol and injecting the ethanolic solution into distilled water (at about 90°) with a fine-tipped pipette. An ethanol blank was prepared at the same time and an equal volume added to all flasks to which the sterol emulsion was not added. The dilutions used gave final concentrations of ethanol in the suspension medium of not greater than 0.05 % (v/v).

The ciliates were incubated with the sterol emulsion or ethanol blank for 1 hr. before the addition of DNP; labelled orthophosphate (^{32}P) was added 15 min. after the DNP, and the organisms removed from the suspension medium 2 hr. after the phosphate addition. The experimental procedures used and the method of calculation were as described by Conner *et al.* (1961).

RESULTS

In all experiments accumulation of orthophosphate by *Tetrahymena pyriformis* W was augmented by the addition of stigmasterol, lowered by DNP, and the DNP effect was mitigated by the stigmasterol (Figs. 1, 2). The increase in orthophosphate accumulation in the presence of a given concentration of stigmasterol was variable, ranging from 2 to 14 % in a series of sixteen experiments. The ethanolic solution had no effect on phosphate accumulation.

The influence of DNP concentration with regard to orthophosphate accumulation is shown in Figs. 1 and 2. At pH 6.5 a concentration of $2.5 \times 10^{-5}\text{M}$ -DNP inhibited approximately 68 % of the phosphate accumulation while at pH 7.5 a DNP concentration of $2 \times 10^{-4}\text{M}$ inhibited to about the same extent. The concentration of undissociated DNP at pH 6.5 is $8.25 \times 10^{-8}\text{M}$ and at pH 7.5 is $6.6 \times 10^{-8}\text{M}$.

The use of a single concentration of DNP and different amounts of stigmasterol resulted in considerable variation in the degree of protection afforded by the stigmasterol, indicating that one or more factors were not adequately controlled in these seven experiments. Figure 3 shows the results of an experiment which most clearly indicated an effect of a sterol concentration gradient. Little difference either in augmentation or in annulment of the DNP inhibition of phosphate accumulation was noted when cholesterol or β -sitosterol were used in place of stigmasterol.

The results of experiments determining the distribution of the accumulated phosphate are given in Table 1. Within a given experiment, the distribution of radiophosphate in the three fractions in terms of percentages remained constant in the presence of DNP and/or stigmasterol, even though an alteration of total radiophosphate accumulation was brought about by these substances. Experiments were performed in duplicate as a gauge of the reliability of the method used; solutions and animals were taken from the same stocks, and time factors were

Table 1. *Inter-experimental range of percentage accumulation and percentage of radio-phosphate recovery in trichloroacetic acid (TCA)-insoluble, TCA-soluble organic and inorganic fractions following treatment of Tetrahymena with DNP (1×10^{-4} M) and stigmasterol (1×10^{-5} M) at pH 7.0 (8 experiments)*

Flask	Phosphate distribution							
	Accumulation (%)		TCA-insoluble (%)		TCA-soluble organic (%)		Inorganic (%)	
	Mean \pm S.D.*	Range	Mean \pm S.D.*	Range	Mean \pm S.D.*	Range	Mean \pm S.D.*	Range
Control	86 \pm 5	78-90	20 \pm 4	15-28	11 \pm 4	8-13	69 \pm 4	64-76
Stigmasterol (1×10^{-5} M)	91 \pm 5	82-95	22 \pm 5	15-30	11 \pm 4	8-14	67 \pm 5	60-75
DNP (1×10^{-4} M)	44 \pm 9	31-53	15 \pm 8	5-28	17 \pm 4	11-23	68 \pm 6	58-77
DNP + stigmasterol	64 \pm 5	56-72	17 \pm 6	12-27	15 \pm 3	10-22	68 \pm 3	63-72

* S.D. = Standard deviation.

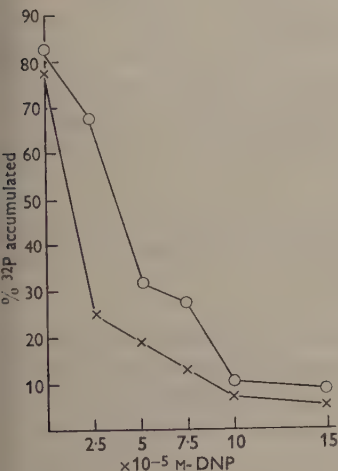


Fig. 1

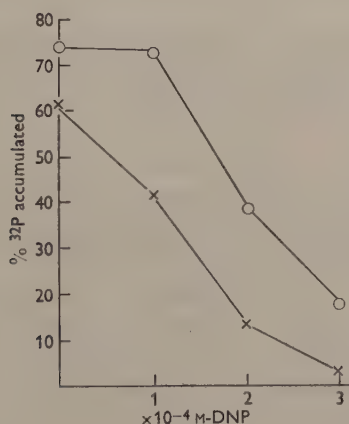


Fig. 2

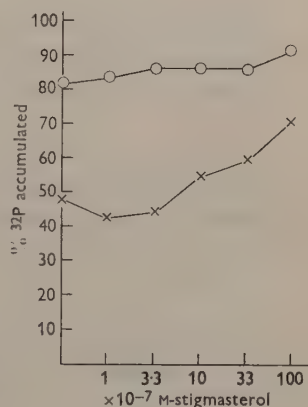


Fig. 3

Fig. 1. The influence of DNP and stigmasterol on phosphate accumulation in *Tetrahymena*. One ml. of organism suspension at an optical density of 0.5 as determined by a Lumetron colorimeter using a 650μ filter was added in a final volume of 10 ml. suspension at pH 6.5. Suspension medium containing DNP (x-x). Suspension medium containing DNP and 1×10^{-5} M-stigmasterol (O-O).

Fig. 2. The influence of DNP and stigmasterol on phosphate accumulation in *Tetrahymena*. Two ml. of organism suspension at optical density of 0.5 in a final volume of 10 ml. Suspension medium at pH 7.5; with DNP (x-x), with DNP and 1×10^{-5} M-stigmasterol (O-O).

Fig. 3. The influence of stigmasterol and 1×10^{-4} M-DNP on phosphate accumulation in *Tetrahymena*. Two ml. organism suspension at optical density 0.5 in a final volume of 10 ml. suspension medium at pH 7.5. Stigmasterol (O-O); stigmasterol and 1×10^{-4} M-DNP (x-x).

Table 2. *Determination of accumulation and distribution of radiophosphate in Tetrahymena in the presence of DNP (1×10^{-4} M) and/or stigmasterol (1×10^{-5} M) at pH 7.0, in duplicate experiments*

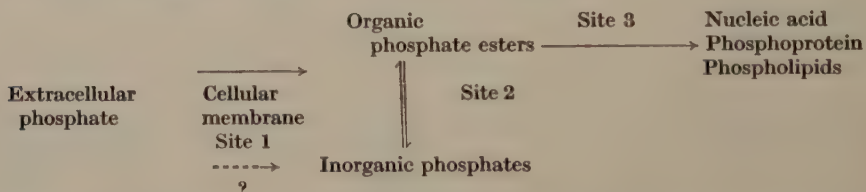
Exp. no.	Flask contents	% removed from medium	TCA insoluble*	TCA soluble*	Inorganic*
1a	Control	87 ± 1.0	22 ± 1.0	10 ± 1.0	68 ± 1.0
	Stigmasterol	95 ± 1.0	30 ± 1.0	8 ± 1.0	62 ± 1.0
	DNP	42 ± 2.0	22 ± 2.5	11 ± 3.0	66 ± 2.0
	DNP + stigmasterol	66 ± 1.5	23 ± 2.0	11 ± 2.0	65 ± 2.0
1b	Control	89 ± 1.0	28 ± 1.5	8 ± 1.5	64 ± 1.5
	Stigmasterol	95 ± 1.5	29 ± 1.0	12 ± 1.0	59 ± 1.5
	DNP	48 ± 1.0	28 ± 2.0	14 ± 2.0	58 ± 2.5
	DNP + stigmasterol	72 ± 1.5	27 ± 2.5	10 ± 3.0	63 ± 3.0
4a	Control	79 ± 1.5	19 ± 1.5	12 ± 1.0	69 ± 1.5
	Stigmasterol	84 ± 1.5	23 ± 1.0	13 ± 1.0	64 ± 1.5
	DNP	32 ± 1.5	16 ± 3.0	14 ± 2.0	70 ± 5.0
	DNP + stigmasterol	60 ± 1.5	15 ± 1.5	16 ± 1.0	67 ± 2.0
4b	Control	78 ± 2.0	19 ± 1.5	14 ± 1.0	67 ± 1.5
	Stigmasterol	82 ± 2.0	19 ± 1.5	12 ± 1.0	69 ± 1.5
	DNP	31 ± 2.0	20 ± 3.5	16 ± 5.0	63 ± 5.0
	DNP + stigmasterol	56 ± 2.0	17 ± 2.0	16 ± 2.0	67 ± 3.0

* Given as percentage of phosphate accumulated.

controlled as closely as possible. Representative duplicate experiments (Table 2) revealed an uncertainty up to 6% in phosphate accumulation and 8% in distribution (see also Kamen & Spiegelman, 1948).

DISCUSSION

In light of available information about phosphate metabolism it is reasonable to propose the following pathway in *Tetrahymena pyriformis* W:



A diminution of phosphate accumulation by DNP can be pictured as a disruption of phosphate metabolism at one or more sites.

Extrapolating from the effects of DNP on phosphate metabolism in mitochondria, lowered ATP production via the oxidative phosphorylation reactions and stimulation of ATPase activity, a decrease in organic phosphate esters and TCA insoluble compounds might be expected, perhaps accompanied by an increase in inorganic phosphate. Loughman & Russell (1957) observed inhibition of radiophosphate accumulation in young barley roots treated with DNP; upon fractionation, they observed greatest diminution in the organic phosphate esters, specifically in the nucleotides, and an increase in orthophosphate. Styrett (1958), working with *Chlorella*, has reported the ATP concentration within the cells to be decreased by high concentrations of DNP.

Similarly, it was thought that stigmasterol might produce an alteration in the pattern of distribution of phosphate which could be interpreted to indicate a site of action. In context of the sterol involvement in the guinea-pig stiffness syndrome, Van Wagtendonk & Wulzen (1951) suggested the steroid might act as a prosthetic group for the enzyme responsible for adenylic acid phosphorylation or possibly as a restraint to ATPase activity.

The pattern found in the *Tetrahymena* experiments shows *no* disturbance in the radiophosphate distribution when the animals are subjected to 1×10^{-4} M-DNP and 1×10^{-5} M stigmasterol at pH 7.0, either singly or in combination. This pattern, too, has an interpretation in terms of the foregoing scheme; DNP acts to prevent transfer of environmental orthophosphate across the cellular membrane to the intracellular 'pool' and apparently the sterols oppose the DNP effect.

It is not possible on the basis of the lack of change in the distribution of accumulated phosphate in the presence of 2:4-dinitrophenol and/or the stigmasterol to distinguish, in *Tetrahymena*, between a direct surface or membrane phenomenon and a general involvement of energy-requiring processes. However, regardless of which mechanism proves to be correct, it is necessary to postulate the sterol and DNP act in a related system. The sterol may act in the process of phosphate accumulation at the level of the cellular membrane or may aid in supplying the energy necessary for accumulation.

Orthophosphate entry seems to many investigators to be connected with metabolic processes at the cell surface. Helder (1952) and Lundegårdh (1955) have proposed mechanisms for surface adsorption in plants. Kamen & Spiegelman (1948), supported by Rothstein & Dennis (1953), argue for a cell-surface esterification as the mechanism for radiophosphate permeation in yeast.

Conner (1961) suggested that phosphate entry in *Tetrahymena* is an active process. DNP could influence this process in one of three ways: (1) by lowering the utilization or increasing the intracellular concentration of orthophosphate; (2) interference with an active accumulation mechanism involved in phosphate entry, directly by combining with a 'carrier'; or (3) indirectly by decreasing the supply of energy required for active transport (Conway, 1955).

Further elucidation of the site and mode of DNP depression and sterol elevation of phosphate accumulation is being attempted by noting if there is sterol interaction with DNP with regard to respiratory elevation (Hamburger & Zeuthen, 1957), respiratory quotient elevation (inhibition of the Pasteur effect) and glycogen loss. An interaction between the sterol and DNP in all of these phenomena would favour a generalized energy hypothesis or interaction in the energy supply mechanisms, while a lack of sterol influence on these DNP-induced disturbances of metabolism would favour a membrane site of action.

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The Type A Phages of *Salmonella typhimurium*: Observations on Temperate Phage and Lysogenesis

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SUMMARY

When a standard suspension of *Salmonella typhimurium* Q1, an indicator strain sensitive to the type A phages recovered from lysogenic strains of this organism, was exposed to concentrations of these phages giving a phage:bacteria ratio of approximately 1:10 (thus ensuring that, with rare exceptions, bacteria became infected with single phage particles) it was found that definite percentages of the bacteria were either lysogenized or productively infected (lysed). These percentages were constant for each particular phage type, but varied widely in the 11 members of the group. It was concluded that each phage consisted of a mixed population of particles, some capable, as single infections, of producing lysogenization (α particles) and others lacking this property (β particles). An increase in the phage:bacteria ratio, resulting in multiple infections of single bacteria, led to an increase in lysogenization at the expense of productive infection. The number of α particles present in any particular concentration of phage was calculated from the figures determined at low phage:bacteria ratios (limit dilution). With some phages, when bacteria became infected with more than one particle by exposure to rising phage concentrations, the number lysogenized was compatible with the hypothesis that α particles are dominant over β particles, and that every bacterium infected by an α particle is lysogenized. This hypothesis was however invalidated by the fact that, with other phages, either the number of bacteria lysogenized was in excess of the available α particles, indicating that bacteria had been lysogenized by infection with two or more β particles, or the number of α particles was grossly in excess of the number of bacteria lysogenized. The implications of these findings are discussed.

Very high concentrations of these temperate phages produced lysis-from-without, and in some cases appeared to induce lysis of bacteria which had first been lysogenized. In all cases in which such experiments were carried out, exposure to low temperatures (20°), to high temperatures (42°), to the salts of certain organic acids, and to anaerobiosis had no significant effect on lysogenization.

INTRODUCTION

The type A phages of *Salmonella typhimurium* (Boyd & Bidwell, 1957) have been studied by us over a number of years, and various details of their lysogenizing properties have been observed, some of which are in agreement with accepted ideas, while others are not. As these closely related phages were all isolated from strains of *S. typhimurium* recovered from infected human beings or contaminated food,

and are therefore members of a natural group, and not artificially produced mutants, the findings are considered of sufficient interest to be placed on record. They may serve to focus attention on the fact that, while remarkable progress has been made in the investigation of the biochemical and biophysical properties and of the genetic aspects of certain 'laboratory' strains of phage, little or no work of a systematic nature has been done in identifying and correlating the phages found, in prophage form, in different species of bacteria. As a consequence, knowledge of bacterial viruses as they occur in nature is fragmentary and elementary, and a vast source of information is virtually untapped.

METHODS

In general, the materials and methods are those which have been described in previous papers (Boyd, 1950; Boyd & Bidwell, 1957). The media used were standardized nutrient agar and Lemco broth, though occasionally, in an endeavour to repeat the results of others, the special media recommended by them were employed. The indicator strain used throughout was *Salmonella typhimurium* Q1 (Boyd, 1956) and the phages were 11 of the 12 types classified by cross-immunity tests (Boyd & Bidwell, 1957) all of which have been adapted to and are maintained and propagated on Q1. Type A4 was not investigated, as repeated and prolonged attempts have failed to recover from this temperate phage the virulent mutant necessary for the experiments. Concentrated suspensions of phage particles— 10^{11} /ml. and over—were prepared by the method described by Liu (1958). All incubations which involved accurate timing were carried out in a Grant waterbath. The EEL nephelometer was used to estimate the turbidity of cultures and suspensions of bacteria. The methods of counting free phage particles in a suspension, which are fundamental to the work, are described in detail in the first part of the paper.

A bacterium is said to be 'lysogenized' when the invading temperate phage is converted to prophage, and 'productively infected' when the phage particle multiplies at the expense of the bacterium to produce a brood of daughter particles. The latter process is for brevity referred to as a 'burst'. Throughout the paper the bacterial indicator is designated Q1, the different phages A1a, A1b, etc., and the lysogenic bacteria Q1 (A1a), Q1 (A1b), etc. The phage particles of a temperate phage are for convenience differentiated by the symbols suggested in an earlier publication (Boyd, 1953). α particles are those which as single infections of a sensitive bacterium produce lysogenesis. β particles are those which, as single infections, produce, not lysogenesis, but a burst. The outcome of such bursts is, of course, a temperate phage-population consisting of both α and β particles. Permanent virulent mutants (Boyd, 1951a) which produce only virulent (lytic) particles (except for an extremely rare back-mutation giving rise to a β particle) are designated γ particles.

RESULTS

Preliminary observations

Counts of bacteria

To make a reliable assessment of the multiplicity of infection, i.e. the number of phage particles adsorbed to each bacterium, accurate counts of bacteria and of the phage particles are required. All bacterial cultures contain non-viable organisms in

numbers which vary according to the technique of preparation. In the experiments recorded in this paper bacterial suspensions were prepared as follows. A nutrient agar slant was inoculated from a stock culture and incubated overnight at 37°. The following morning the required number of tubes of broth was inoculated from this culture and kept for 3 hr. at 37°, by which time the organisms were well advanced in the logarithmic phase of growth. These cultures were then pooled and diluted with broth to an opacity shown by previous trials to contain approximately 10^8 viable bacteria/ml. when further diluted with 1/10 of its volume of broth or phage concentrate. In every experiment a control count of viable bacteria was carried out.

Using suspensions prepared in this way, counts of all the bacteria, viable and non-viable, were made by means of a Thoma haemocytometer, 0.02 mm. in depth, while counts of viable bacteria were calculated from colony counts on agar plates which had been flooded with 0.5 ml. of a diluted suspension. The results, shown in Table 1,

Table 1. *Comparison of total counts and viable counts of bacterial suspensions*

Expt.	Total bacteria* per ml.	Viable bacteria† per ml.	% viable	% non-viable
1	1.9×10^8	1.04×10^8	52.5	47.5
2	2.5×10^8	1.45×10^8	57.5	42.5
3	1.74×10^8	9.9×10^7	57.0	43.0
4	1.82×10^8	9.2×10^7	69.0	31.0
5	1.51×10^8	9.1×10^7	60.0	40.0
6	1.47×10^8	1.04×10^8	70.0	30.0
7	1.57×10^8	1.17×10^8	74.0	26.0
8	1.51×10^8	1.05×10^8	69.0	31.0
9	1.79×10^8	1.14×10^8	64.0	36.0
Average	1.7×10^8	1.08×10^8	63.5	36.5

* Total counts were made by means of a Thoma haemocytometer, 0.02 mm. in depth, using dark-field illumination.

† Viable counts were made from suitable dilutions of the bacterial suspension flooded in 0.5 ml. quantities over the surface of at least 3 plates of nutrient agar.

reveal the fact that roughly one-third of the bacteria are non-viable. It is not possible to separate viable and non-viable bacteria in order to assess their respective adsorptive properties, but it may be assumed that the adsorptive capacity of non-viable bacteria will not differ greatly from that of bacteria killed by minimal heat. In Table 2 the adsorption of phage by a living culture is compared with that of a culture heated to 58.5° for 30 min. There is little significant difference. The slightly decreased adsorption shown by the dead bacteria may be explained by the fact that they are non-motile and hence may have made slightly fewer collisions with the phage particles. This suggests that viable and non-viable bacteria in a culture are likely to have similar adsorptive properties.

The presence of relatively large numbers of non-viable bacteria in the suspensions used in the experiments to be described does not in fact vitiate the results. As identical suspensions were used in counting the phage particles, it follows that an equivalent proportion (approximately one-third) of the phage particles were 'lost' in the counting process by becoming adsorbed to non-viable bacteria. The phage:bacteria ratio and similar calculations are therefore correct when given in terms of the viable bacteria in a suspension, provided a suspension prepared in the same way was used in counting the phage particles. The total bacterial count and

the total phage count are in fact half as much again as the figures indicate, but this can be disregarded, for both sides of the equation are equally affected. The practical point which emerges is that each experiment on lysogenization or lysis must be controlled, not by a total count of bacteria by whatever means calculated, but by a count of the viable bacteria in the suspension used in the particular experiment.

Method of counting virulent (γ) phage particles

Phage particles which develop only by the 'productive' or lytic cycle, and do not produce lysogenesis, were counted, not by the usual pour-plate method (Hershey, Kalmanson & Bronfenbrenner, 1943, slightly modified by Adams, 1959), but by the

Table 2. *Comparison of the adsorptive properties of living and heat-killed suspensions of Q1*

The bacterial culture gave a count of viable organisms of approximately 10^8 /ml. Phage was added to give the ratios indicated, and the mixtures incubated for 15 min. at 37° . Thereafter the mixtures were at once diluted to prevent further adsorption, the living bacteria killed by heating to 70° for 30 min. and counts made of the plaque-forming free phage particles.

Phage:bacteria ratio		...	15:1	1.5:1	0.15:1
Percentage of phage particles adsorbed by	Culture of living bacteria		67.5	94	98.3
	Bacteria killed by heating		65.5	92	95.2
	to 58.5° for 30 min.				

Table 3. *Comparison of plaque-counting techniques*

Decimal dilutions of concentrated phage A1b were counted by three methods. Each figure in the table is an average of 2 counts. The surface count, measuring drops with the Agla micrometer syringe, is consistently higher than Adams's pour-plate method using 0.7% agar for the 'layer'. The third method, using 1.2% agar, gives an even lower count and shows that, the greater the viscosity of the agar, the greater is the loss caused by the mixture sticking to the tube from which it is poured.

	Surface count	Adams's pour-plate (0.7% agar layer)	Pour-plate (1.2% agar layer)
		Plaques/ml.	
1	1.25×10^{11}	1.0×10^{11}	8.1×10^{10}
2	1.23×10^{10}	1.17×10^{10}	8.15×10^9
3	1.21×10^9	1.02×10^9	9.0×10^8
4	1.28×10^8	1.24×10^8	8.55×10^7

surface-count method (Boyd, 1950). After a preliminary rough titration, the phage concentrate was accurately diluted to a point where it contained, in terms of the preliminary titration, 10^3 particles/ml., and, with an Agla micrometer syringe, 5 drops of exactly 0.01 ml. of this diluted concentrate were placed at different points on a 'lawn' of Q1 (i.e. the dried surface of a 10 cm. Petri dish of nutrient agar previously flooded with a broth culture of Q1). The drops were spread, but not to the point of coalescence, by gently rocking the plate, which was then incubated overnight. In the morning the plaques were counted, and from this count, multiplied by the appropriate figure to correct the dilution, the number of particles in the original concentrate was calculated. This method is simpler and quicker than the pour-plate method, and gives counts which are about 10% higher (Table 3). This is

because, in the pour-plate method, bacteria and phage are mixed with soft agar in a tube before being poured on to a plate of agar of normal consistency. Inevitably some of the agar mixture sticks to the sides of the test tube, and so is lost to the count, whereas when using the Agla syringe, a drop of the exact size is ejected from the square-cut needle without loss.

Method of counting temperate phage particles

When bacteria are mixed with temperate phage diluted to such an extent that each organism is infected by only one phage particle, the infected bacteria are either rendered lysogenic (by α particles) or are productively infected (by β particles). If such a phage-bacteria mixture, suitably diluted, is spread on a lawn of indicator bacteria, the productively infected bacteria will produce turbid plaques of normal size, but, as will be seen later, only a relatively small number of the lysogenized bacteria will produce plaques, most of which will be of small size. To make an accurate count it is therefore necessary to use a technique which will reveal both lysogenized and productively infected bacteria.

The α particles in a temperate phage were counted as follows. A preliminary count of the plaque-forming particles was made by the method just described for γ particles. (For convenience this preliminary count of a temperate phage, which is used as a starting point in certain of the experiments, is called a plaque count. In a high-titre concentrate the plaque count was approximately 10^{11} /ml.) The concentrate was then accurately diluted to give 10^8 plaque-forming particles/ml. One ml. of this dilution was added to 9 ml. of a suspension of Q1, giving a final count of 10^7 plaque-forming particles and approximately 10^8 bacteria/ml., i.e. a ratio of about 1:10. This is regarded as 'limit' dilution, the dilution at which each phage particle will in general infect a single bacterium. The phage-bacteria mixture was immediately placed in a waterbath at 37° for 10 min. to allow adsorption to take place, and thereafter rapidly diluted $10^{-5} \times 1/5$ in broth. Volumes of 0.5 ml. of this dilution were flooded on to plates of nutrient agar which had been impregnated with 10^9 particles/ml. of the virulent (γ) mutant of the phage under investigation. On such a medium only bacteria which had been lysogenized by the temperate phage multiplied and formed colonies: productively infected bacteria disintegrated, and all non-lysogenized (sensitive) bacteria were destroyed. After overnight incubation, the average number of colonies per plate $\times 10^8$ gave the number of lysogenizing particles per ml. of the phage-bacteria mixture, and a further calculation in terms of the preliminary dilution gave the count in the original concentrate. The accuracy of this method of estimating lysogenized bacteria has been tested by plating equal quantities of phage-bacteria mixture on (a) nutrient agar and (b) nutrient agar impregnated with virulent phage. Each colony developing on (a) was tested independently for lysogenesis. The totals of the lysogenic colonies on (a) and (b) in a series of experiments with graded phage concentrations are shown in Fig. 1. If anything, the percentage of lysogenization revealed by the impregnated plate technique was lower than in the control, a finding which indicates that multiple infection with γ particles does not produce lysogenization. In this and all subsequent experiments, the percentage of bacteria lysogenized—or lysed—was calculated in terms of the count of viable bacteria in the control culture which was included in every experiment.

The second half of the count—the estimation of the number of particles which give productive infection—was a continuation of the same experiment. Another portion of the phage-bacteria mixture was diluted in broth $10^{-7} \times 1/16$, and distributed in quantities of 0.4 ml. in 200 small sterile test-tubes. This gave an average of 1 bacterium per 4 tubes, a distribution which made it improbable that more than one bacterium would find its way into any one tube. As, at the phage dilution used, only

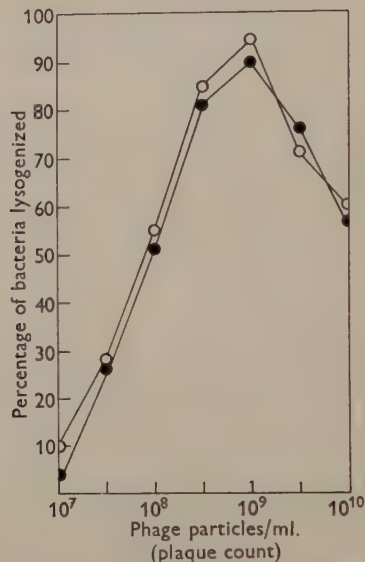


Fig. 1. Comparison of two methods of estimating lysogenization. Q1, at a concentration of 10^8 bacteria/ml., was exposed to graded concentrations of phage A1b, and incubated at 37° for 10 min. The preparations were then diluted $10^{-5} \times 1/5$ and equal quantities from each plated on (1) nutrient agar and (2) nutrient agar impregnated with 10^9 particles/ml. of A1b γ . All colonies which developed on (1) were tested for lysogenesis. Only lysogenic colonies developed on (2). ○, Percentage of bacteria lysogenized, calculated from (1); ●, percentage of bacteria lysogenized, calculated from (2).

about 1 in 10 of these bacteria was phage-infected, the chance of more than one productively infected bacterium being in any one tube can be disregarded. The tubes were then incubated at 37° for 90 min. Thereafter a loopful from each was placed on a numbered section of a lawn of Q1, and incubated overnight. The presence of multiple plaques on any one section indicated a burst. If a section showed only one plaque, which might be produced by a residual free phage particle, a larger sample was tested. With the above technique and dilutions, the number of tubes showing bursts multiplied by 2×10^6 gave the number of β particles/ml. of phage-bacteria mixture.

Lysogenization and productive infection at limit dilution in the Type A phages

The results of a series of counts of phages A1b and A2d by the methods described are recorded in Tables 4 and 5. These are two phages used in many of the experiments to be described, as they are representative types having respectively high and low lysogenizing properties. In both cases the sum of the α and β particles,

which will be taken as the true count, was higher than the plaque count, though this difference was more marked in A1*b* than in A2*d*. Counts of particles carried out in this way at limit dilution gave relatively constant and repeatable results, both in the same batch and in different batches of any one phage. These relative proportions of α and β particles appear therefore to be a stable character of each particular phage when prepared and tested by the methods described.

Table 4. *Comparison of counts of particles in temperate phage A1b made by enumerating lysogenic bacteria and bursts, and by plaque count*

The counts of lysogenic bacteria and bursts were made from a standard phage-bacteria mixture containing 1.1×10^7 particles/ml. (as estimated by plaque count).

Expt.	Lysogenic bacteria	Bursts	Sum of lysogenic bacteria and bursts	Plaque count
1	8.25×10^6	1.0×10^7	1.825×10^7	—
2	9.5×10^6	6.0×10^6	1.55×10^7	—
3	9.0×10^6	4.0×10^6	1.3×10^7	—
4	9.25×10^6	8.0×10^6	1.725×10^7	—
Average	9.0×10^6	7.0×10^6	1.6×10^7	1.1×10^7

Note. The discrepancy between the plaque count and the bursts is attributable to the fact that newly lysogenized bacteria are unstable, and frequently, after 3 or 4 divisions, produce daughter cells in which productive development occurs. The liberation of free phage in this way produces, in the fully incubated plate, plaques of a size related to their time of origin, early ones being large and late ones small.

Table 5. *Comparison of counts of particles in temperate phage A2d made by enumerating lysogenic bacteria and bursts, and by plaque count*

The counts of lysogenic bacteria and bursts were made from a phage-bacteria mixture containing 1.0×10^7 particles/ml. (as estimated by plaque count).

Expt.	Lysogenic bacteria	Bursts	Sum of lysogenic bacteria and bursts	Plaque count
1	1.85×10^6	9.6×10^6	1.15×10^7	—
2	1.95×10^6	1.1×10^7	1.2×10^7	—
3	1.15×10^6	1.34×10^7	1.5×10^7	—
4	1.3×10^6	1.0×10^7	1.13×10^7	—
5	1.1×10^6	1.2×10^7	1.31×10^7	—
Average	1.47×10^6	1.12×10^7	1.26×10^7	1.0×10^7

Counts of all 11 types of A phage were carried out by these methods at limit dilution. The average of several counts—at least two—is given in Fig. 2, which shows the relative proportions of α and β particles. The wide range of variation is a notable feature.

Observations on multiplicity and lysogenization

It has been pointed out (Boyd, 1951*b*) and confirmed (Lwoff, Kaplan & Ritz, 1954) that when a sensitive culture is exposed to temperate phage, the percentage of bacteria which becomes lysogenized increases when the bacteria are infected by more than one phage particle. Such multiplicity of infection and subsequent lysogenization is governed partly by the proximity of the bacteria and phage particles,

and partly by the availability of phage particles. Table 6 records an experiment showing the extent of lysogenization when a culture of Q1 containing 10^8 organisms/ml. was exposed to widely spaced concentrations of A1b. The peak of lysogenization was reached when the concentration of phage was 10^9 particles/ml., a phage:bacteria ratio of 10:1.

The phage:bacteria ratio is not in itself the dominant factor. Decreasing the number of bacteria, and so increasing the phage:bacteria ratio does not increase the degree of lysogenization (Table 7).

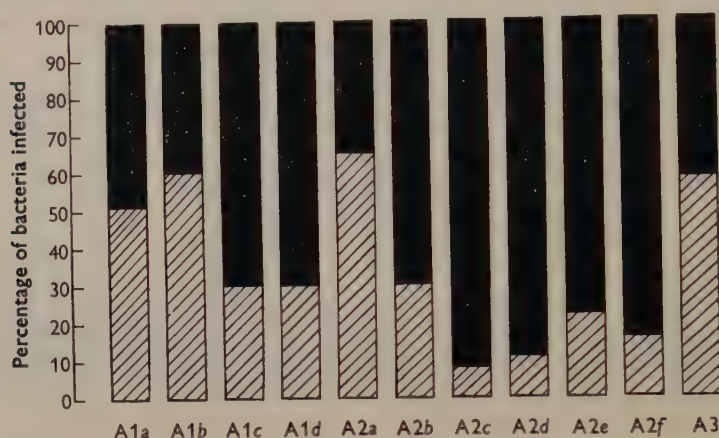


Fig. 2. Relative proportions of α and β particles in the A phages. The α (lysogenizing) and β (productive infection) particles in each phage were counted, at limit dilution, by the method described. ▨, α particles; ■, β particles.

Table 6. *Relationship of phage concentration and lysogenization (Q1 and A1b)*

Mixtures of Q1 and A1b were prepared by the methods described, and incubated at 37° for 10 min. to allow adsorption to occur. Thereafter they were appropriately diluted and plated on agar impregnated with A1b γ . The percentage of bacteria which had become lysogenized was calculated from the number of colonies which developed. The figures given are the average of several experiments.

Concentration of phage particles/ml.	1.0×10^7	3.1×10^7	1×10^8	3.1×10^8	1.0×10^9	3.1×10^9	1.0×10^{10}
Phage:bacteria ratio (approximate)	0.1:1	0.31:1	1:1	3.1:1	10:1	31:1	100:1
Percentage of bacteria lysogenized	5.0	18.0	38.0	77.0	87.0	77.0	65.5

On the other hand, when the bacterial concentration is lowered, lower phage concentrations, within limits, give an equally high degree of lysogenization, as the bacteria have the opportunity to come in contact with and adsorb a greater number of particles (Table 8).

These results emphasize the need for a standard technique, if consistent and comparable results are to be obtained. It is for this reason that bacterial suspensions containing approximately 10^8 viable organisms/ml. have been used in all experiments.

*Lysogenesis and productive development at different phage concentrations**Phage A1 b*

Experiments were carried out in which Q1 was exposed to different concentrations of A1 *b*. Thereafter estimations were made of the percentage of bacteria lysogenized, the percentage of bacteria which underwent productive development, the percentage which escaped phage infection, and the percentage 'unaccounted for' (in low phage concentrations a small and variable number attributable to unavoidable experimental error). Figure 3 gives in graphic form the results of a series of experiments with A1 *b*, using widely spaced phage concentrations, and Fig. 4, the results of a more closely spaced series.

Table 7. *Lysogenization with constant phage concentration and decreasing bacterial concentration*

Decreasing concentrations of Q1 were exposed to a constant concentration of temperate A1 *b*, giving an increasing phage:bacteria ratio. This produced no significant increase in the number of bacteria lysogenized.

Phage particles/ml.	7.5×10^8	7.5×10^8	7.5×10^8	7.5×10^8	7.5×10^8	7.5×10^8	7.5×10^8
Viable bacteria/ml.	9.3×10^7	4.65×10^7	2.325×10^7	1.162×10^7	5.81×10^6	2.9×10^6	1.45×10^6
Phage:bacteria ratio	7.5:1	15:1	30:1	60:1	120:1	240:1	480:1
Lysogenized bacteria/ml.	7.5×10^7	3.93×10^7	2.05×10^7	9.46×10^6	4.42×10^6	2.4×10^6	1.1×10^6
% lysogenized	80	84	88	81	76	82	77

Table 8. *Lysogenization when bacterial concentration is low*

Against lower bacterial concentrations, lower phage concentrations produce a higher percentage of lysogenization.

Bacteria/ml.	Phage particles/ml. (A1b)			
	10^7	10^8	10^9	10^{10}
	% bacteria lysogenized			
10^8	8.5	51	85	55
10^6	40.0	84	83.5	58.5

The first column in Fig. 3, in which the phage:bacteria ratio is 1:10, represents limit dilution, and shows the respective proportions of α and β particles in this particular sample of phage. Lysogenization increased at the expense of productive development up to a phage concentration of 4×10^8 , and maintained a high level in subsequent concentrations up to 10^9 . Beyond this there was a progressive increase both in the percentage of bacteria which ceased to be viable for reasons other than productive development of phage, and, unexpectedly, in the percentage of bacteria in which phage underwent productive development. These increases were balanced by a decrease in the percentage of lysogenized bacteria and by the absence of unaffected bacteria.

Except at limit dilution, the phage:bacteria ratio does not give any exact indication of the number of particles adsorbed by the infected bacteria. Although in ratios of 1:1 and under, over 95 % of the added phage was adsorbed before the calculation was obscured by phage production from lysing bacteria (Fig. 5) the number of bacteria infected was less than the number of phage particles available (Figs. 3, 4),

and it can be assumed that the excess phage was shared by the infected bacteria in accordance with the Poisson distribution. Thus in ratios as low as 0.31:1 some of the bacteria adsorbed more than one particle.

Phage A2d

As A2d, when examined at limit dilution, has been found to contain a lower percentage of α particles and a higher percentage of β particles than A1b (Fig. 2), the action of this phage on Q1 at different concentrations was investigated in the

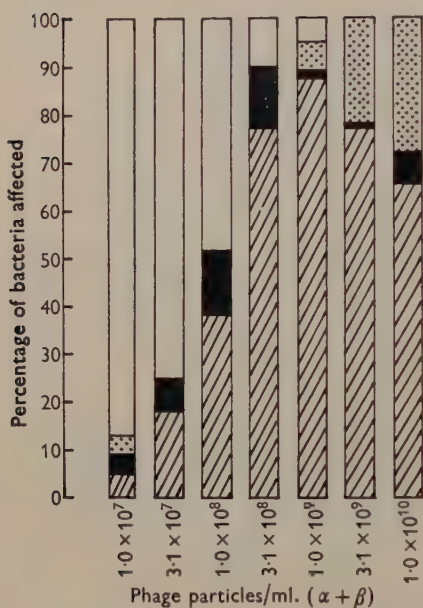


Fig. 3

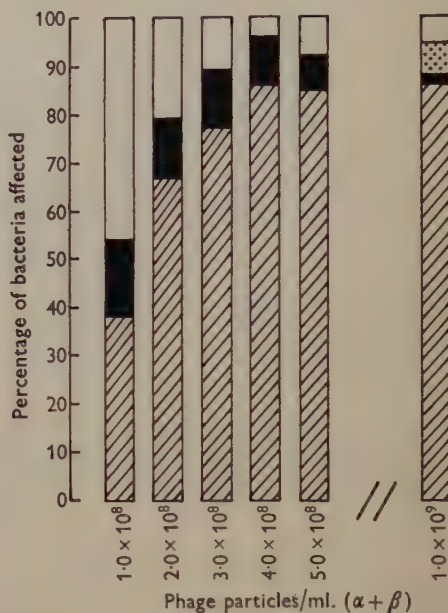


Fig. 4

Fig. 3. Action of widely spaced rising concentrations of A1b on Q1. Q1 (10^8 bacteria/ml.) was exposed for 10 min. at 37° to the phage concentrations indicated. Thereafter the percentage of lysogenized bacteria and the percentage of bursts was determined by the methods described. The percentages of 'bacteria uninfected' and bacteria 'lost' were calculated from the viable count of a control bacterial suspension to which no phage was added. In this figure, the phage concentrations are based on true counts, i.e. the sum of α and β particles calculated at limit dilution. Each column is the average of 2 or more experiments. \square , Lysogenies; \blacksquare , bursts; \boxtimes , bacteria lost; \square , bacteria uninfected.

Fig. 4. Minimum concentration of A1b needed for maximum lysogenization of Q1. A similar experiment to that recorded in Fig. 3, but with closely spaced phage concentrations. Same key as for Fig. 3.

same way as was A1b. The results are shown graphically in Fig. 6, and can be seen to conform to the same general pattern. Maximum lysogenization, which however was at a lower level than with A1b, occurred at a similar phage concentration and increased in the same way at the expense of productive infection. The percentage of bursts was higher at all concentrations than in the case of A1b.

Relationship of the number of α particles to the number of bacteria lysogenized when exposed to different concentrations of temperate phage

Suspensions of all 11 type A phages were prepared at limit dilution and in accurately measured ascending concentrations thereof. Standard suspensions of Q1 were exposed to these phages, and the number of bacteria lysogenized in a given unit (actually 0.5 ml. of a $10^{-5} \times 1/5$ dilution of the original mixture) were estimated in the usual way. The number of bacteria lysogenized at limit dilution revealed the

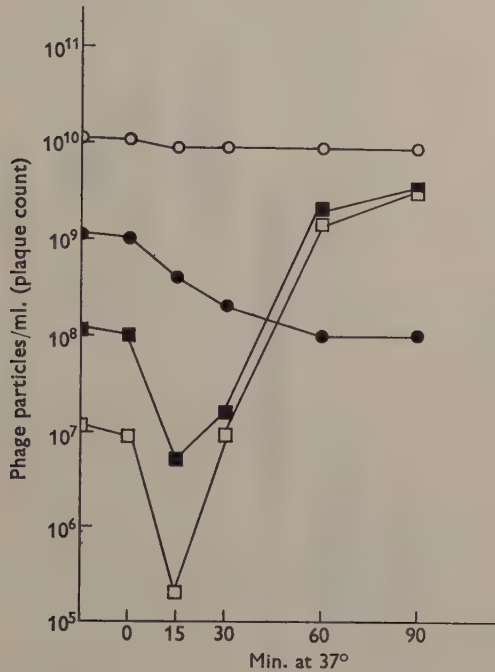


Fig. 5. Adsorption of A1b on Q1. A1b was added to suspensions of Q1 (10^8 bacteria/ml.) to give the concentrations indicated. Samples were removed at intervals and immediately heated to 70° for 30 min. to kill the bacteria. The curves are plaque counts of free phage particles. Phage concentration: ○, 10^{10} /ml.; ●, 10^9 /ml.; ■, 10^8 /ml.; □, 10^7 /ml.

number of α particles in this particular unit. From this the number of 'available' particles in units of the higher concentrations was calculated. In Fig. 7 the number of bacteria actually lysogenized is shown in relation to the number of α particles available. It will be seen that in some cases the α particles were in excess of the bacteria lysogenized, while in other cases the reverse held good. These results will be analysed in the Discussion.

The well-marked variations in lysogenization resulting from exposure to the different phages were not due to a smaller number of bacteria becoming phage-infected because of defective adsorption. Experiments on a more limited range of concentrations, in which both lysogenization and productive development were estimated (Table 9), showed that, while variations occurred in the total number of bacteria infected, these were not related to the degree of lysogenization. Thus A2b

produced a relatively low rate of lysogenization associated with a high percentage of infection, while A2c had an opposite reaction in the top ratio, namely a higher degree of lysogenization with a much lower percentage of infection. The exceptionally low percentage of infected bacteria in the 10:1 ratio of A2c was associated with a high percentage of 'bacteria lost', and may be attributable to unusually active 'lysis-from-without'.

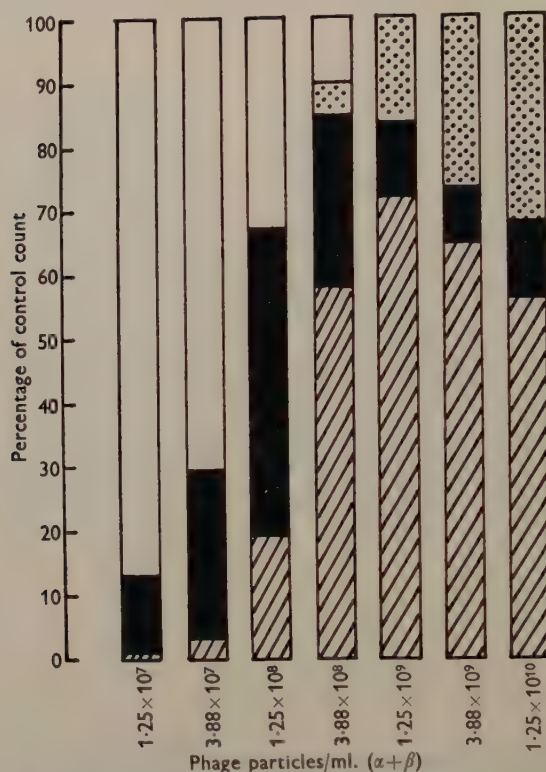


Fig. 6. Action of widely spaced concentrations of A2d on Q1. A similar experiment to that recorded in Fig. 3, but with A2d. ▨, Lysogenics; ■, bursts; ▤, bacteria lost; □, bacteria uninfected.

Lethal action of high concentrations of temperate phage

Andrewes & Elford (1932) drew attention to the fact that an excess of virulent phage brought about destruction of a number of bacteria without any increase of free phage—a phenomenon which they called 'lysis-from-without'. This phenomenon has frequently been described in relation to virulent phage. Lieb (1953) mentions results which suggest similar action in the case of temperate phage R, on *Escherichia coli* K 12 S. It can be seen from Fig. 3 that, in one of the lower phage concentrations, a small percentage of bacteria is 'lost'. This is a not uncommon experimental error attributable to minor variations in the viable counts of the control and the test suspensions. In higher concentrations—from 10^9 onwards—there is a progressive increase in the number of 'missing' organisms (Figs. 3, 6). Although there is no

Phage:bacteria
(Approximate ratio)

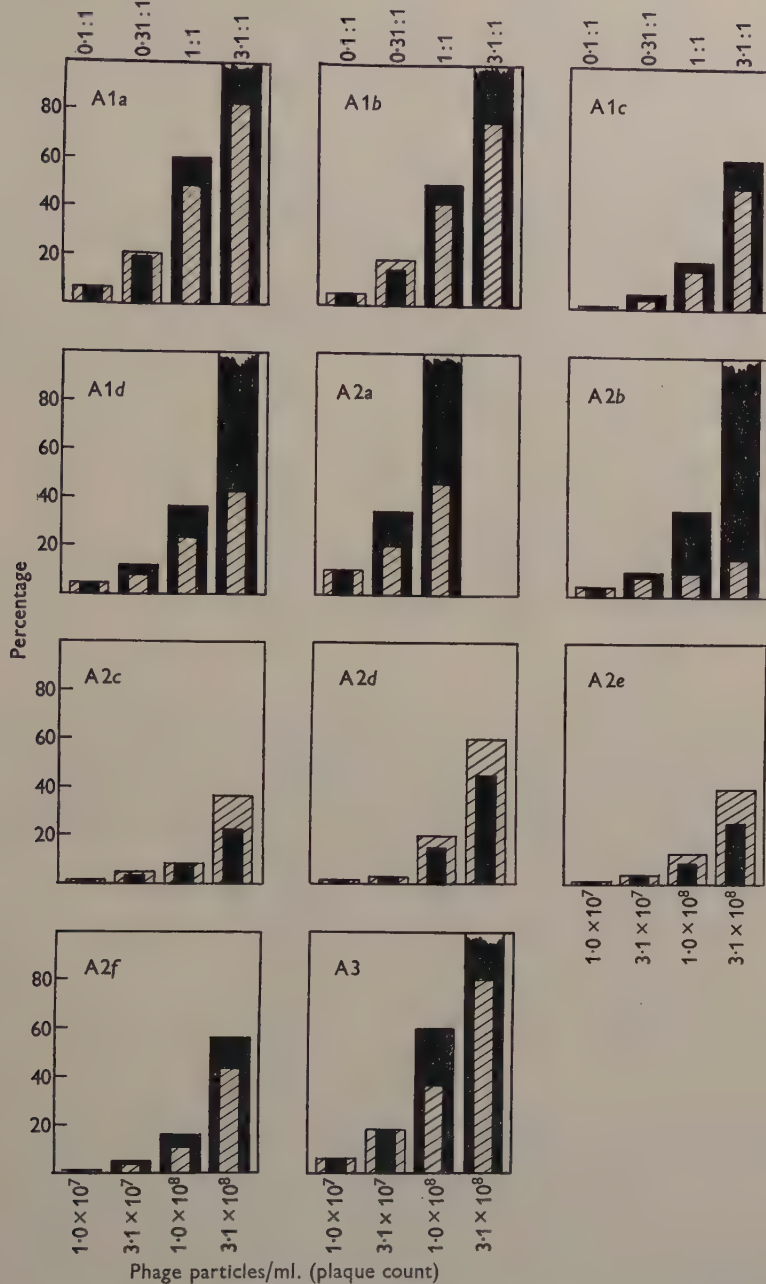


Fig. 7. Relationship of α particles to lysogenesis. Suspensions of Q1 (10^8 bacteria/ml.) were exposed to graded concentrations of the phages and the percentage of bacteria lysogenized estimated by the usual technique. The percentage of α particles 'available' in each phage concentration was calculated from the figure obtained at limit dilution. For convenience in comparing results the lower figure in each column is shown within the higher. ■, α particles in this phage concentration; ▨, bacteria lysogenized.

definite proof that these bacteria were destroyed by lysis-from-without, the picture resembles so closely that found in similar experiments with virulent phage that it leaves little doubt that the bacteria have been killed in this way. A control experiment using corresponding concentrations of the original phage lysate which had been filtered through a gradacol membrane to remove phage particles gave negative results.

Table 9. *Percentage of bacteria infected at different ratios*

The percentage of lysogenies and bursts at different phage:bacteria ratios was estimated by the standard technique. The ratios are in terms of the plaque count. Except in the case of A2c, there are no gross variations in the total number of bacteria infected at the different ratios.

Phage:bacteria ratio	%	Phages containing high proportion of α particles		Phages containing medium proportion of α particles		Phages containing low proportion of α particles	
		A1b	A2a	A1d	A2b	A2c	A2d
0.1:1	Lysogenies	7.9	8.7	3.6	3.6	0.8	1.25
	Bursts	5.5	4.6	8.8	8.5	9.3	11
	Total infected	13.4	13.3	12.4	12.1	10.1	12.25
1:1	Lysogenies	49.25	41.25	22.3	15.7	10	19
	Bursts	14.75	30	36.0	58.0	48	48
	Total infected	64.0	71.25	58.3	73.7	58	67
10:1	Lysogenies	89	80.5	70.0	41.5	61.0	72
	Bursts	1.5	17.7	20.0	51.0	7.3	12
	Total infected	90.5	98.2	90.0	92.5	68.3	84

Table 10. *Lysis-from-without produced by A1b γ*

Q1 was exposed to A1b γ in the concentrations recorded, incubated at 37° for 10 min., diluted $10^{-7} \times 1/16$, and distributed in 0.4 ml. quantities in 200 tubes. At the same time appropriate quantities were plated to estimate viable survivors. After the tubes had been incubated at 37° for 2 hr., a loopful from each was spotted on a lawn of Q1 to ascertain the number of tubes in which bursts had occurred.

Phage particles/ml.	Percentage of		
	Bursts	Surviving bacteria	'Missing' bacteria
10^7	10.6	85.4	4.0
10^8	62.5	23.6	13.9
10^9	82.0	1.3	16.7
10^{10}	63.0	1.0	36

Confirmatory evidence of the lysis-from-without phenomenon was provided by experiments in which Q1 was exposed, not to temperate phage A1b, but to its virulent mutant, A1b γ , which does not produce lysogenesis. The effect of different phage concentrations on the percentage of bacteria in which the phage underwent productive development was investigated by the usual technique. The results of one series of experiments are given in Table 10. The percentage of bacteria which supported productive development was approximately the same as the percentage of bacteria which were lysogenized by temperate phage, and at high phage concentrations there was a similar decrease in the number of infected bacteria, presumably due to lysis-from-without.

Productive infection in high concentrations

An unexpected and constant finding in experiments with A1*b*, which was present but less obvious in the case of A2*d*, was that productive infection reached its minimum at a phage concentration of around 10^9 particles/ml. and tended to increase in the higher concentrations (Figs. 3, 6). It would therefore appear, in a heavy multiple infection, either that lysogenesis is not established, and productive development supervenes, or that the immunity which lysogenesis affords breaks down. This problem was explored by exposing an established strain of Q1 (A1*b*) to the action of different concentrations of A1*b*, and examining for bursts in the usual way. With a phage concentration of 10^9 particles/ml., no productive infection occurred, while at $10^9 \times 5$, 6 %, and at 10^{10} , 8 % of the bacteria showed productive infection.

In these experiments there was no evidence of lysis-from-without, although A1*b* is as freely adsorbed to Q1 (A1*b*) as to Q1. Counts of viable bacteria made before and after exposure to the phage showed no significant variation other than the decrease attributable to the bursts.

Q1 (A2*d*) is immune to A1*b* in concentrations of 10^9 particles/ml. When Q1 (A2*d*) was exposed to 10^{10} particles A1*b*/ml. some 6 % of the cells burst, liberating particles of A1*b* and A2*d* in approximately equal numbers. Thus it would seem that, although Q1 (A2*d*) is immune to low concentrations of A1*b*, a high multiple infection with A1*b* induces Q1 (A2*d*). The significance of these results will be discussed later.

Influence of temperature on lysogenization and productive development

Bertani & Nice (1954) found that incubation of infected bacteria at a temperature of 20° for 2 hr. blocked the productive development of phage P1, and also of its virulent mutant, whereas the proportion of infected bacteria which became lysogenized was much higher at a temperature of 20° than at 37°. Temperate phage P2 did not react in this way. This observation was investigated, burst experiments being carried out with A1*b* at a phage:bacteria ratio of 0.75:1. One portion was incubated at 37°, the other at 20°. In one the percentage of bursts was 17.2 and in the other 18. Thus there was no significant difference and no suppression of productive development as found in the case of P1. Similar results were obtained in further experiments using different phage concentrations, in which both lysogenesis and productive development were estimated. Like P2, A1*b* is therefore unaffected in this way by incubation at low temperature.

Lieb (1953) reports that, in the case of *Escherichia coli* and phage λ , the temperature to which the bacteria are exposed during the first 1 to 1½ hr. after infection has a predominant influence on the proportion of lysogenics in clones, i.e. in the progeny of what are believed to be single cells. This is a different type of phenomenon from that described by Bertani, but it was considered of interest to find out if exposure to a high temperature affected the lytic or productive response of Q1 infected with A1*b*. The standard technique was used and the results are given in Table 11. The higher temperature reduced the number of bacteria which showed the effects of phage infection, presumably due to restricted bacterial growth, but the relative proportions of bacteria lysogenized and bacteria productively infected did not vary significantly from the control.

Influence of organic acids on lysogenization

Lwoff *et al.* (1954) using A1a and *Salmonella typhimurium* 1404 as indicator, report that specified concentrations of the potassium salts of certain organic acids (citric, malic, oxaloacetic, malonic, pyruvic, lactic, and pyrophosphoric) increase productive infection from 3 to 5 times, presumably at the expense of lysogenic infection. Early experiments carried out with the same phage and indicator some years ago failed to confirm this claim, the only significant action observed, with the methods and media then in use, being some interference with adsorption and consequent slight reduction in the number of bacteria which became phage infected.

Table 11. *Influence of high temperature on lysogenization*

The A1b:Q1 ratio in all cases was 0.75:1. Lysogenization and productive infection were ascertained by the standard technique.

	Temperature of incubation	
	37°	42°
% lysogenized	35.2	24*
% productively infected	13.5	9.3

* The figures under 42° are the average of 2 experiments.

Table 12. *Influence of citrate and malonate on lysogenization*

Lwoff's synthetic medium was used throughout. The concentration of phage A1b in all experiments was 7.5×10^7 /ml. Lysogenesis and productive infection were ascertained by the standard technique.

	% of bacteria	
	Lysogenized	Productively infected
Synthetic medium control	25.5	15.5
Synthetic medium + potassium citrate 10^{-2} M	31.5	12.1
Synthetic medium + potassium malonate 2×10^{-2} M	34	12.2

The action of potassium citrate and potassium malonate has been re-investigated with A1b and Q1, but using Lwoff's synthetic medium instead of Lemco broth. The results confirmed those given by the earlier experiments. There was no gross variation in productive development, and only a slight increase in lysogenization (Table 12). Lwoff's synthetic medium was used throughout and in the control the degree of lysogenization was less than is found in Lemco broth. The concentration of A1b in all experiments was 7.5×10^7 /ml. In further experiments with A1a and 1404 at a 5:1 ratio, in which only lysogenization was estimated, there was a slight reduction in the percentage of organisms lysogenized (41.5% control, 36.5% with potassium citrate M/10⁻², 33.25% with potassium malonate M/2 $\times 10^{-2}$). In view of the results given by the early experiments, this can be attributed to some interference with adsorption. In the absence of a marked decrease in lysogenesis, no significant increase in productive infection can have occurred.

Influence of anaerobiosis on lysogenization

It has been stated that anaerobiosis decreases the proportion of lysogenic responses (Lwoff, 1953). No such phenomenon was observed in experiments with A1*b* and A2*d* (Table 13). Two sets of plates were inoculated with aliquots of phage-infected bacterial suspensions prepared by the standard technique, and one set was incubated aerobically, the other anaerobically. The colonies which developed on the plates incubated anaerobically were considerably smaller than those which grew under aerobic conditions, but numerically there was no difference. Two further experiments similar to the above were carried out, in which anaerobiosis was maintained at all stages of the experiment, and not merely during incubation of the plates. Once again the only difference was in the size of the 'anaerobic' colonies, which were much smaller than those in the control.

Table 13. *Influence of anaerobiosis on lysogenization*

The assessment of lysogenization was made by the standard technique. The controls, which are not shown in the table, were normal.

	% of bacteria lysogenized	
	Incubated aerobically	Incubated anaerobically
A1 <i>b</i> :Q1 = 0.75:1	29	28.3
A2 <i>d</i> :Q1 = 1:1	12	12

Influence of chloramphenicol on lysogenization

In contrast to these negative results, chloramphenicol had a well-marked influence on lysogenization. This is a reaction of considerable complexity which it is hoped to make the subject of a separate paper.

DISCUSSION

An essential prerequisite for the study of the processes of lysogenization is a reliable technique for estimating the percentage of bacteria lysogenized and the percentage productively infected when a culture of sensitive bacteria is exposed to the action of temperate phage. The methods which have been elaborated for this purpose are given in detail in the first part of this communication, and do not require further discussion. They are considered to be reliable and accurate, and have been found to give repeatable results. The method of estimating the numbers of productively infected bacteria by counting plaques ('lytic centres') has proved unreliable with the systems we are investigating. We have found that newly lysogenized Q1 is relatively unstable, and may, after a few divisions, throw off a daughter cell in which the phage undergoes productive development. If this occurs before the bacteria in the sensitive lawn have multiplied to any appreciable extent, the free phage thus liberated forms a plaque which is smaller than but otherwise indistinguishable from the plaque produced by an organism productively infected *ab initio*. We have not used as a routine the method described by Levine (1957) turning on the use of gal⁺ and gal⁻ strains of *Salmonella typhimurium*, as the gal⁻ indicator strains available at the time this work was done were found to contain B

phages (Boyd, 1950) and were insensitive to several of the type A phages of the series.

When Q1 was exposed to one of these temperate phages at a concentration sufficiently low to ensure that only single infection occurred it was found that some of the infected bacteria were lysogenized and others were productively infected. The proportions of lysogenized and productively infected bacteria were, within the limits of experimental error, constant in repeated tests of the same batch of phage and in other batches of the same phage made by the same technique but at a different time. Thus it can be concluded (*a*) that a temperate phage consists of a mixture of particles, some (α particles) endowed with the property of producing lysogenesis, and others (β particles) incapable, as single infections, of doing so and going on instead to productive development, and (*b*) that the two types of particle, under standard conditions, are present in relatively constant proportions in each temperate phage.

Eleven of the 12 type A temperate phages (Fig. 2) were investigated in this way (type A4 had to be excluded as no virulent mutant of this phage has been isolated). The proportions of α and β particles varied greatly from phage to phage, though each type behaved consistently. In some the α particles were equal in number to or even more numerous than β particles, in others they constituted as little as 8% of the total. These accurate estimates of the two varieties of particle in any one temperate phage, and the demonstration of their presence in varying proportions in the 11 members of the series, provide a useful means of studying certain of the problems of lysogenization.

It has been suggested (Parry & Edwards, 1953) that the decision towards lysogenesis or productive development of the phage turns on the interplay of two factors, namely varying degrees of virulence on the part of the phage particles and varying degrees of resistance to lysis on the part of the bacteria. However, in view of the wide variation in the pattern of lysogenesis and lysis shown by the 11 phages when tested against the same bacterial suspension, it is obvious that under these conditions bacterial resistance plays a secondary role in determining the outcome of phage infection. Clearly the dominating factor is in the phage particle, and is a constant feature of each particular type. This point is well demonstrated by the results given by A1*b* and A2*d* (Figs. 3, 6), in which the differences recorded have been confirmed in experiments repeated at long intervals of time and with different batches of reagents.

In previous papers (Boyd, 1951*b*, 1953), the existence of α and β particles was deduced from less convincing experimental evidence, and it was suggested that the explanation of the multiplicity phenomenon might lie in a dominance of α particles over β particles. When bacteria were exposed to rising concentrations of phage, increasing numbers would be infected with several particles, some with β particles only, some with α and β particles. In the latter case the pattern of development, i.e. lysogenesis, would be imposed by the dominant α particle, thus producing a higher degree of lysogenization. This idea has been independently explored by Prell & Prell (1959). Working with phage P22, which is derived from the same lysogenic bacterium as A1*b* and gives similar reactions, Prell & Prell found that with low phage:bacteria ratios (0.3:1) the probability per phage is 0.57 for lysogenization and 0.43 for lysis. This is in good agreement with the finding recorded in Fig. 3.

Prell & Prell develop the speculative theory outlined above, and conclude "in multi-complexes the immunity induced by one of the superinfecting phages is superimposed upon the one-hit lytic infection causing the percentage of lysogenization increasing with multiplicity".

Bertani (1960) suggests that calculations of the numbers of bacteria lysogenized by single hits may be complicated by the presence of 'doublets' in the culture—doublets being 'physiologically independent, but incompletely separated, sister bacteria'. He points out that, in a stationary culture, if one half of a doublet is hit by a particle which multiplies productively, the second half will be heavily infected when the first half bursts, and so will run a good chance of being lysogenized by this multiplicity. He points out rightly that this is more likely to increase the proportion of bacteria lysogenized when the phage:bacteria ratio is low. In our experiments the bacteria were actively motile throughout. In the case of A1*b* the proportion of lysogenies and lytics is such that any part played by doublets in increasing lysogenization would be difficult to assess. But from the results given by A2*c* and A2*d*, where at limit dilution approximately 10 % or less of the particles give lysogenesis, while 90 % or more give lysis, it can be seen that, with the technique used, the occurrence of late lysogenesis resulting from the lytic infection of one half of a doublet must be relatively rare. As the bacterial suspensions in all these experiments were prepared by standard methods, and therefore contained the same proportion of doublets, it would appear that our results have not been significantly affected by this possibility.

The results recorded graphically in Figs. 3, 4 and 6 show that with rising phage concentrations and hence rising phage:bacteria ratios, resulting in multiple infection of bacteria by phage particles, the maximum degree of lysogenization is reached in phage concentrations between 4×10^8 and 10^9 particles/ml., at which concentrations two-thirds or more of the phage is adsorbed in 15 min. (Fig. 5). (In the case of A1*b*, calculating from the protocols from which Fig. 5 was constructed, at a concentration of 10^7 (ratio 0.1:1) over 98 % of the phage was adsorbed; at 10^8 (ratio 1:1) 95 % and at 10^9 (ratio 10:1) 67.5 %. Similar experiments with other phages gave results of the same order.)

At limit dilution (for convenience, a plaque count:bacteria ratio of 1:10) the relative proportions of α and β particles per unit of phage of known concentration are revealed (Fig. 7, Table 9). These figures enable the actual number of α and β particles in the accurately measured higher phage concentrations to be calculated. In these higher concentrations the total number of infected bacteria (i.e. both lysogenized and lysed) is less than the total number of phage particles which have been adsorbed. It follows therefore that some infected bacteria have taken up more than one phage particle: the expected distribution can be calculated from the Poisson formula. The total number of bacteria infected by different members of the series and at different concentrations shows some variation (Table 9), but this variation bears no obvious relationship to the proportions of bacteria lysogenized or lysed.

These principles have been employed in constructing the graphs in Fig. 7. The starting point in each case is the first column, which shows the number of bacteria lysogenized by, and hence the number of α particles in, a unit of the phage at limit dilution. The figures recorded in the subsequent columns show the results given by

identical units of accurately measured rising concentrations of the same phage. The hatched columns show the bacteria lysogenized, while the solid columns show the number of α particles in the unit, calculated from the figure given at limit dilution. To make the results obvious at a glance the lower column of the two is enclosed in the higher.

Three different types of response are revealed in Fig. 7.

(1) The number of lysogenized bacteria exceeds the number of available α particles. This reaction is seen in the second column (phage concentration 3.1×10^7) of A1a and A1b, and throughout in A2c, A2d, and A2e.

(2) The number of lysogenized bacteria falls significantly below the number of available α particles. This is well marked in the case of A2b, less so in A1d and A2a.

(3) The number of lysogenized bacteria, though lower than the number of α particles, represents approximately the number of bacteria which, in terms of the Poisson distribution, would have been hit by one or more α particles. This is seen in certain concentrations of A1a, A1b, A1c, A2f and A3.

It must be reiterated that as the same bacterium (Q1) was used throughout these experiments, and as the different phages were adapted to and propagated on this organism, such variations as occur are attributable only to the phage, and thus as far as bacterial resistance is concerned, the different experiments act as controls, one for the other.

The results given by the phages listed under (1) disprove the speculative hypothesis (Boyd, 1951b) that lysogenesis results only when an α particle infects a bacterium, either as a single infection or in association with β particles. In the higher concentrations of A2c, A2d, and A2e, the number of bacteria which were lysogenized exceeded the number of α particles in the unit of phage to which they were exposed. On the other hand, in these higher phage concentrations a number of bacteria were infected by two or more particles, which in most cases were β particles. It can be assumed that single infections with a β particle will produce the same result (a burst) in high as in low phage concentrations, and that the only significant difference between infected bacteria in the high phage concentrations and infected bacteria in the limit phage concentrations was that some of the former were infected by more than one particle. It therefore would appear that in these systems multiple infection, irrespective of whether the particles are α or β , can bring about lysogenesis, and that when two or more β particles enter one bacterium, they act together in some way to produce the conditions essential for lysogenization. A possible but speculative explanation is that α particles possess a component which determines conditions leading to lysogenization: that in individual β particles this component is incomplete, but that when the infective material of two or more β particles enters a bacterium, the effect is additive, and the necessary conditions for lysogenization are produced.

This finding has something in common with observations made by Kaiser (1957) and Levine (1957). Kaiser studied independently occurring clear plaque-forming mutants of phage, among which he recognized three different phenotypes. Mixed infection with a pair of phenotypically different mutants, each of which lysogenized poorly or not at all, produced a high frequency of lysogenization characteristic of infection with the wild type. The surviving bacteria were lysogenic for one or both of the infecting types of phages. Levine worked with clear plaque-forming mutants

of P22 (A1b). He found that, when sensitive bacteria were exposed to a mixture of a virulent mutant, which of itself gave no lysogenesis, and a very weak temperate phage, which gave a very low degree of lysogenization, these two phages combined in multiple infections produced a high degree of lysogenization. These of course are true mutants with genetic deficiencies: if kept uncontaminated, they reproduce their own type indefinitely. β particles are not mutants in this sense, as the progeny of single infection with a β particle is a mixed brood of α and β particles in the standard proportions characteristic of the parent type.

In the case of the phages listed under (2), the position is reversed. At limit dilution the percentage of α particles is average to high. When bacteria are exposed to higher phage concentrations, the number of lysogenized bacteria falls below, and in some cases far below, the number of available α particles, even when 'doubling up' is taken into consideration. This is not due to defective adsorption (Table 9). In the case of these phages, multiplicity would appear to antagonize lysogenization instead of increasing it. Whether or not this is due to the presence of a restraining component in the β particles or to some external factor, so far unrecognized, is a question which cannot at present be answered.

The phages listed under (3) give results which are mainly in conformity with the original theory that lysogenization follows infection with a dominant α particle, and if no other phage types but these had been examined (the type on which the theory was based was A1a) a convincing case could have been built up.

An alternative explanation of these findings which has been propounded is that each phage consists, not of a mixed population of particles with differing properties of lysogenization, but of a uniform population in which there is a certain probability that any one phage particle can give rise to a lysogenic clone: that this probability is a constant for each phage: and that the probability is doubled in a doubly infected cell, trebled in a trebly infected cell and so on. When multiplicity values are calculated according to the Poisson formula, and adjusted to the probability revealed by the percentages of lysogenesis and lysis at limit dilution, a set of theoretical figures can be constructed. The results set out in Fig. 7 show a considerable degree of agreement with these theoretical figures (much better agreement than the discarded 'dominant α particle' theory) but in all cases show some deviation, and in one or two cases gross deviation.

Apart from the by no means perfect fit of theoretical figures and results this hypothesis is open to criticism on at least two scores. What are the factors which determine the certain probability that any one particle can give rise to a lysogenic clone, bearing in mind that this probability varies widely in these closely related but nevertheless distinct phage types? The bacteria are prepared by standard methods, and while individual organisms may have varying characters they present the same front in all the experiments. The varying degrees of lysogenesis and lysis produced by infection with the different phage types cannot therefore be attributed to the bacteria. Before either lysis or lysogenesis occurs, the hazards of adsorption and penetration lie behind: these cannot explain the phenomenon. The 'certain probability' must therefore result from events inside each bacterium after it has been penetrated by the infective material of a phage particle. The bacteria, although they may vary individually, e.g. in age in terms of the last division, are *en masse* alike in all the experiments. The infective material from each phage particle, according

to this hypothesis, is uniform for each phage type. When A1*b* is the infecting phage, an average of six out of ten of these identical units succeed in circumventing some undefined barrier, and become integrated in the bacterial chromosome: four of the ten fail to do so. In the case of A2*d*, only one out of ten of the invading particles reaches this goal. As all the particles in each phage are held to be alike, it follows that all, both A1*b* and A2*d*, are capable of producing lysogenesis. The varying number of successful lysogenizations must therefore be attributed to the chance evasion of some barrier or inhibiting mechanism within the bacterium, a chance evasion in which the different phage types have varying but consistent degrees of success. When the issues involved are examined in this way, the probability hypothesis becomes unconvincing, the more so as the existence of a mechanism capable of preventing lysogenization is speculative and its nature is undefined.

The second criticism of the probability theory lies in the fact that none of the experiments, admittedly limited in number, involving environmental changes of the bacterium-phage complex, made any significant difference to the percentages of lysogenesis or lysis.

On balance, the conception of a phage population, mixed in the sense that its members, though genetically identical, are endowed to a varying degree with some factor essential for lysogenization, is more in keeping with the available experimental evidence.

The fallacy of drawing conclusions of a general nature from the results given by a single system is heavily underlined by the results of these experiments, and, as a corollary, the necessity for widening the field of investigation is clearly demonstrated. Apart from showing that in certain cases lysogenization can result from multiple infection with β particles, these findings are of interest mainly because they show the complexity of the multiplicity phenomenon. They provide no solution capable of general application.

The experiments with A1*b* and A2*d* (Figs. 3, 6) demonstrate two other responses in bacteria exposed to very high concentrations of these temperate phages which are worthy of brief mention.

The first is a lethal response corresponding to the phenomenon which has been observed in the case of virulent phages and has been described as lysis-from-without (Andrewes & Elford, 1932). The possibility of this reaction being produced by a weak bactericide which is lethal only when in high concentration cannot be altogether disregarded, although rendered improbable by the fact that the lethal property is removed by passing the lysate through a gradacol filter with pores sufficiently small to hold back the phage particles. The alternative explanation, a weakening of the bacterial wall by the tail-enzymes of the phage particles (Puck & Lee, 1954) is in keeping with the accepted basis of this phenomenon in other systems.

The second is the occurrence of an increasing number of bursts, particularly in the case of A1*b* (Fig. 3). This observation prompted an experiment in which an established strain of Q1 (A1*b*) was exposed to a high concentration of its homologous temperate phage, A1*b*, to which in low concentration it is immune. A number of bursts resulted. An established strain of Q1 (A2*d*) was then exposed to a high concentration of the heterologous temperate phage, A1*b*, to which in low concentrations it is immune. Again a number of bursts occurred, the resulting free particles being of both types, A1*b* and A2*d*. With this evidence that a high concentration of

temperate phage can 'induce' a lysogenic bacterium which is immune to the same phage in lower concentrations, it seems possible that the bursts under consideration arise from a two-stage process—an early lysogenization followed by induction.

With the exception of chloramphenicol, various chemical and physical agencies which have been found by other workers to affect lysogenesis have shown no specific or significant action on the systems which we have tested. Neither heat, nor cold, nor anaerobiosis, nor organic acids have had any effect other than a non-specific action attributable to interference with bacterial growth and multiplication.

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The Effect of *N*-Ethylmaleimide on the Radiation Sensitivity of Bacteria

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SUMMARY

The sensitization of *Escherichia coli* strain B/r to γ radiation was shown to take place within a few minutes of the addition of *N*-ethylmaleimide. Sensitization was demonstrated with as little as 0.0001 M *N*-ethylmaleimide, but a much higher concentration (> 0.002 M) was necessary for any bactericidal effect. The *N*-ethylmaleimide had to be present during irradiation in order to be effective. *Staphylococcus aureus* and a *Pseudomonas* sp. but not spores of *Bacillus subtilis*, were also sensitized by *N*-ethylmaleimide. The *Pseudomonas* sp. was only sensitized under anoxic conditions; this may indicate that, with this organism, *N*-ethylmaleimide and oxygen acted competitively in enhancing radiation damage. Possible mechanisms of sensitization are discussed.

INTRODUCTION

Nitric oxide and oxygen both enhance radiation damage when present in suspensions of the vegetative forms of bacteria during irradiation (Howard-Flanders, 1957; Hollaender, Stapleton & Martin, 1951). Oxygen is also effective with spores, although nitric oxide has been found to have a net protective effect on them (Powers, Webb & Kaleta, 1960). Until recently little attention had been paid to other substances which might sensitize bacteria to radiation. Bridges (1960) reported that *N*-ethylmaleimide (NEM), when present during irradiation, was able to increase the lethal action of γ radiation on *Escherichia coli*, an effect more pronounced under anoxic conditions. The present paper records further study of the action of NEM on certain micro-organisms.

METHODS

Chemical. *N*-ethylmaleimide (NEM) was obtained from the Aldrich Chemical Co. Inc. (Milwaukee, Wisconsin, U.S.A.). It was dissolved in sterile 0.067 M-phosphate buffer (pH 7) to make a 0.01 M-solution which was kept at 3-5° and diluted as required. Sterilization of this solution was found to be unnecessary.

Micro-organisms. *Escherichia coli* strain B/r (in the sequel this strain will be referred to as *Escherichia coli* B/r), obtained in 1958 from Miss Tikvah Alper (Hammersmith Hospital), was used throughout this work. The *Pseudomonas* sp. was isolated from a chicken carcass by Dr Margaret Thornley (Low Temperature Research Station, Cambridge), and *Staphylococcus aureus* was no. 7447 of the National Collection of Type Cultures. Cultures (20-24 hr.) on nutrient agar slopes

were washed off and shaken with 10 ml. 0.067 M-phosphate buffer (pH 7) and 1 ml. of this suspension added to 9 ml. of the test solution before irradiation. Spores of *Bacillus subtilis* were obtained by allowing 24 hr. cultures on nutrient agar plates to stand on the bench for several days. The growth was then washed off with buffer, centrifuged, resuspended in buffer, and the suspension heated in an 80° water bath for 10 min. The spore suspensions were shaken with glass beads to break up clumps and were used within a few hours of preparation. NEM was added 4–6 min. before irradiation except where otherwise stated.

Estimation of radiation damage. The ability of bacteria to form visible colonies on the surface of nutrient agar (Oxoid) plates incubated at 30° was used as the criterion of viability. Colonies usually appeared within 24 hr. although after treatment with a high concentration of NEM subsequent growth was slow and colonies continued to appear for up to six days.

Irradiation technique. A ^{60}Co γ radiation source giving a dose rate of 300 krad./hr. was used at room temperature. Samples (10 ml.) of test suspension were irradiated in glass vessels, and air or oxygen-free nitrogen was bubbled vigorously through the suspensions during the irradiation. With nitrogen, bubbling was begun 6 min. before irradiation, to remove dissolved oxygen.

RESULTS

Sensitization of Escherichia coli B/r

The survival curves for *Escherichia coli* B/r in the presence and absence of 0.001 M-NEM are shown in Fig. 1; it can be seen that a pronounced sensitization occurred. Where the shape of survival curves of sensitized and control bacteria is the same, the extent of any sensitization may be described by the 'dose modifying factor' (d.m.f.) which is the ratio of the dose of radiation needed to cause a given degree of damage in the absence of the sensitizer to that required in its presence. For example, the presence of oxygen in an air-saturated suspension results in a d.m.f. of 2.6 with *E. coli* B/r. Under anoxic conditions, 0.001 M-NEM was equivalent to a d.m.f. of 2.0, but sensitization was not so marked under aerated conditions where the d.m.f. was 1.3. There was no enhancement of radiation damage when NEM (to 0.001 M) was added immediately after irradiation in buffer (Fig. 1). Bacteria initially incubated with 0.0005 M-NEM for 5 min. and then diluted 100-fold or treated with

Table 1. *The effect of removing N-ethylmaleimide (NEM) immediately before irradiation on the radiation sensitivity of Escherichia coli strain B/r*

NEM was used at 0.0005 M; irradiations were performed under anoxic conditions.

		Mean survival after 30 krad. (%)
Untreated controls	—	33.1
NEM added 10 min. before irradiation	(a) Not removed	16.1
	(b) Removed by addition of excess cysteine 5 min. before irradiation	31.6
	(c) Removed by 1/100 dilution 5 min. before irradiation	32.8

excess (0.001M) cysteine to remove any unreacted NEM were not sensitized to subsequent irradiation in absence of oxygen (Table 1).

The greater effect of NEM under anoxic conditions might have been due to a requirement for a period of incubation with the compound before irradiation, since under anoxic conditions there was always nitrogen bubbling for 6 min. before each incremental dose of radiation. Various times of pre-incubation under aerobic conditions were therefore tried. It can be seen from Fig. 2 that a definite sensitization was observed with pre-incubation for as little as 1 min. There was no increase in sensitization when the pre-incubation period was increased from 5 to 15 min. The difference in pre-incubation time does not therefore explain the difference in effect as between aerated and anoxic conditions. The rapidity with which sensitization was established would seem to exclude the possibility that any gross changes in the physiological state of the cells before irradiation were involved although it does not exclude the possible involvement of the reaction with cellular -SH groups.

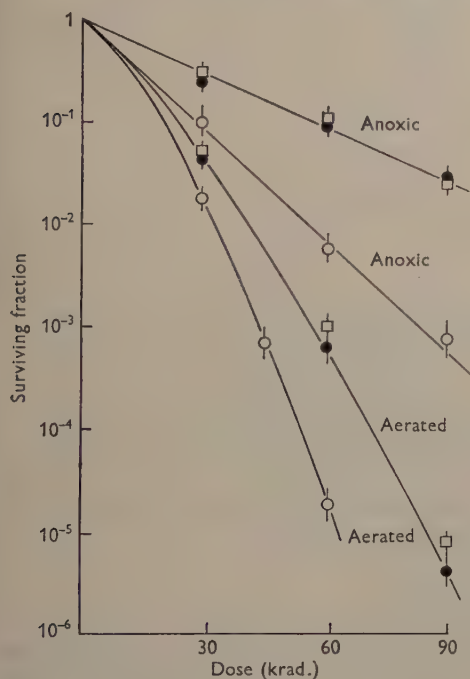


Fig. 1

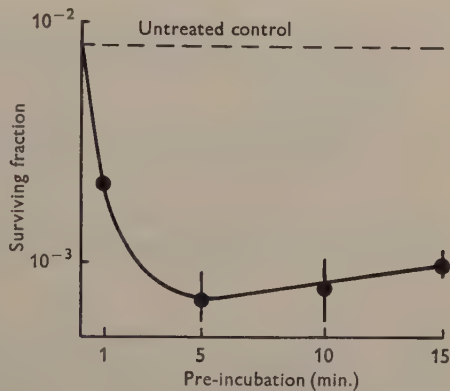


Fig. 2

Fig. 1. Effect of 0.001M-NEM on the radiation sensitivity of *Escherichia coli* strain B/r. ●, No NEM; ○, NEM present during irradiation; □, NEM added immediately after irradiation.

Fig. 2. Effect of pre-incubation with 0.001M-NEM on surviving fraction of *Escherichia coli* strain B/r after 45 krad. under aerated conditions.

Toxicity of NEM

Figure 3 shows the toxicity of NEM at varying concentrations. As judged by the ability of the treated organisms to form visible colonies on nutrient agar, NEM was non-toxic up to 0.001M, while at 0.01M there was an appreciable lethal effect. Although treatment with 0.001M-NEM for 1 hr. did not prevent the subsequent

formation of colonies, they appeared more slowly than the controls. Colonies appeared at the normal rate, however, when 0.001 M-homocysteine was present in the plating medium. The presence of this sulphhydryl compound did not decrease the toxicity of NEM or the degree of sensitization.

Effect of concentration

The influence of concentration on sensitization under anoxic conditions is given in Fig. 4; a similar curve was obtained under aerated conditions. There appears to be a threshold at about 0.00005 M-NEM, below which no significant sensitization was observed. Above this concentration the effect increased rapidly and reached a maximum at 0.0003 M. Above this there was apparently a very slight decrease in sensitization.

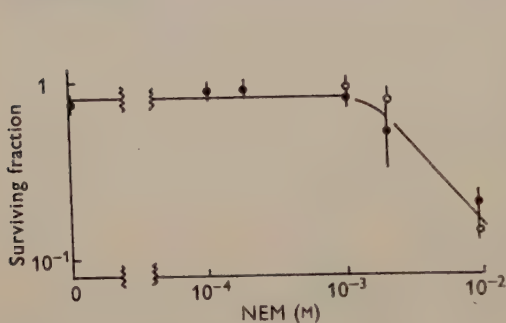


Fig. 3

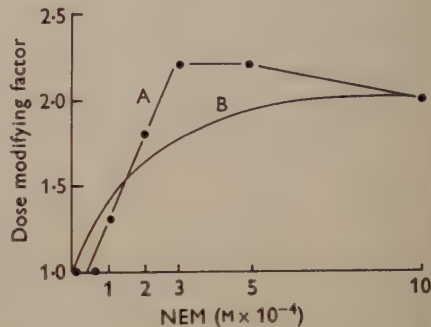


Fig. 4

Fig. 3. Effect of incubation with NEM for 1 hr. on viability of *Escherichia coli* strain B/r. ●, Plate counts on nutrient agar; ○, Plate counts on nutrient agar containing 0.001 M-homocysteine.

Fig. 4. Effect of concentration of NEM on surviving fraction of *Escherichia coli* strain B/r after 60 krad. under anoxic conditions. A, experimental curve; B, theoretical curve according to equation of Alper & Howard-Flanders (1956) where $m = 2.2$, $K = 175 \mu\text{M}$.

Alper & Howard-Flanders (1956), in studying the oxygen effect, represented the effect of concentration by the following formula:

$$\text{d.m.f.} = \frac{m[\text{O}_2] + K}{[\text{O}_2] + K},$$

where m is the maximum d.m.f. and K is the concentration of oxygen at which half the maximum sensitization is attained. Applying these terms to NEM under anoxic conditions, m may be assumed to be 2.2 and K , 175 μM . A curve plotted from these values in such an equation is shown in Fig. 4; the curve does not characterize the experimental points. The effect of the concentration of NEM appears therefore to be significantly different from that of oxygen.

Experiments with other organisms

The effect of 0.001 M-NEM on the radiation sensitivity of *Staphylococcus aureus* is shown in Fig. 5. It was essentially similar to that obtained with *Escherichia coli* B/r, the d.m.f. values being 1.55 under anoxic conditions and 1.17 in air. The *Pseudomonas* sp. differed from *S. aureus* and *E. coli* B/r in that although no sensitization was produced by 0.001 M-NEM under aerated conditions, there was a very marked effect

under anoxic conditions where the d.m.f. was 2.0 (Fig. 6). It is of some interest that the effect of oxygen on radiation sensitivity was large with this pseudomonad, the slope of the survival curve under air being 4.25 times as great as that under nitrogen. Spores of *Bacillus subtilis* were not sensitized to radiation by 0.001 M-NEM under anoxic or aerated conditions. This might have been due to the failure of NEM to penetrate the spore wall but no experiments were carried out to verify this. NEM was not toxic to any of the above organisms at 0.001 M.

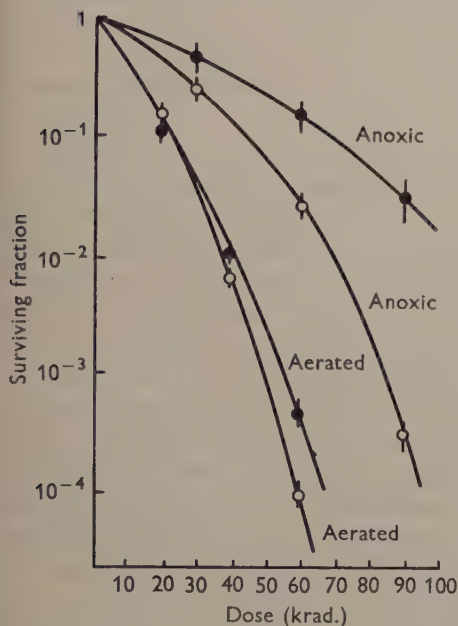


Fig. 5

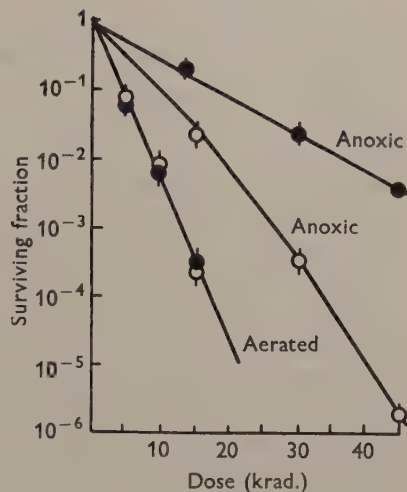


Fig. 6

Fig. 5. Effect of 0.001 M-NEM on the radiation sensitivity of *Staphylococcus aureus*. ●, Control; ○, NEM.

Fig. 6. Effect of 0.001 M-NEM on the radiation sensitivity of *Pseudomonas* sp. ●, Control; ○, NEM.

DISCUSSION

Sensitization is apparently dependent upon the simultaneous action of NEM and radiation since organisms incubated with 0.0005 M-NEM for 10 min. and then diluted 1/100 or treated with excess cysteine (to 0.001 M) to remove unreacted NEM, were not sensitized. This eliminates the two most obvious explanations of the mechanism of sensitization, i.e. that NEM combines with cellular -SH groups and in some way renders them more sensitive to the action of radiation, or that NEM combines with naturally occurring -SH protective agents. Sulphydryl groups on biological molecules have been classified as 'freely reacting', 'sluggish' and 'masked' (Barron, 1951). All the freely reacting -SH groups are likely to react with NEM within a very few minutes of its addition (Roberts & Rouser, 1958), and since the reaction is irreversible they will lose their biological activity. That some physiological damage does occur seems indicated by the finding that the endogenous respiration of *Escherichia coli* B/r is 70 % inhibited by 0.001 M-NEM (Bridges & Marples, un-

published observation). This damage to cellular $-SH$ groups is obviously not lethal since the organisms are able to form colonies when diluted out and plated. Although colony formation is slower than usual it may be speeded by up the addition of $-SH$ groups to the medium in the form of homocysteine. Such a treatment does not, however, overcome the sensitization.

The fact that a certain amount of NEM reacts with cellular $-SH$ groups probably accounts for the threshold observed in the concentration curve. Presumably a certain concentration is required before there is sufficient uncombined NEM remaining to cause sensitization. The organisms used in these experiments were not washed and might be expected to have had a fairly high concentration of $-SH$ compounds arising from the nutrient medium, in addition to those normally present within the organism.

A possible explanation of the data is that the NEM reacts with some molecule which is in a short-lived reactive state induced by the radiation. This molecule, in the absence of NEM, stands a certain chance of being restored or changed to an innocuous form. A similar model has been proposed for the enhancing effect of oxygen (Alper & Howard-Flanders, 1956; Alper, 1958). In this case the oxygen is envisaged as undergoing a 'metionic' reaction with a free radical centre, probably on a carbon atom, resulting in the formation of a peroxide radical. It is quite possible that NEM could react across its double bond with a carbon-free radical centre. Such reactions are known in the radiation-induced cross-linking of rubber which is catalysed by dimaleimides (Vale & Roberts, 1960). A further possibility is that NEM reacts with $-SH$ groups (or possibly $-S$ -free radicals) which result from the radiation-induced breakage of $-S-S-$ bonds, necessary for the functional state of some proteins and other molecules of biological importance. Combination of NEM with either of the sulphur atoms could prevent the $-S-S-$ bond reforming and result in the loss of the biological integrity of the molecule.

Since oxygen partially (completely, with the *Pseudomonas* sp.) inhibited the action of NEM, it is conceivable that both compete for the same free radical centres. The study of sensitizing agents such as NEM may therefore be of use in understanding the oxygen effect and, ultimately, the nature of the lethal damage which radiation causes within the cell.

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The Nature of the Widespread Soil Fungistasis

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SUMMARY

Fungistasis of Conover loam, muck, and hardwood forest soils to conidia of *Glomerella cingulata*, *Fusarium oxysporum* f. *lycopersici*, and *Penicillium frequentans* was demonstrated by indirect methods such as agar disks, double agar layer plates, and cellophan folds, as well as by direct placement of spores on or in soils. All attempts to extract toxic substances from soil with water or organic solvents failed. Fungistatic volatile substances could not be demonstrated in soil. Redox, pH, and osmotic conditions were not responsible for soil fungistasis. Various lines of evidence led to the conclusion that the so-called widespread soil fungistasis as observed by indirect methods is the result of production of antibiotics by soil microbes growing on the surface of the assay media, and is not due to a reserve of toxic substances in soils. The possibility is suggested that individual fungus spores serve as nutrient microsubstrates in soil and stimulate the rapid growth of soil microbes on their surface or in their immediate vicinity, and that this results in the production of sufficient fungistatic substances to prevent spore germination. Preliminary evidence in support of this suggestion are results with concentrated 50 % ethanol washings from teliospores of *Ustilago zeae* which markedly stimulated growth of mixed soil microbes and of pure cultures of *Streptomyces* sp. and *Pseudomonas* sp., known antibiotic producers, in agar media.

INTRODUCTION

Spores of fungi which are able to germinate readily in distilled water or nutrient substrates fail to germinate in natural soils. Since the work on this problem by Dobbs & Hinson (1953), many authors have demonstrated fungistasis in different soils by using spores of numerous fungi (Chinn, 1953; Hessayon, 1953; Jefferys & Hemming, 1953; Jackson, 1958*a*; Lockwood, 1959; Stover, 1958). This apparently widespread fungistasis has been attributed to diffusible toxic factors in soil, and appears to coexist with biological activity since soils sterilized by heat or fumigants are not inhibitory (Dobbs & Hinson, 1953; Jackson, 1958*b*; Lockwood, 1959; Stover, 1958). Toxicity to fungi has been restored to autoclaved soil by inoculating with a specific bacterium (Park, 1956*a*) or with various actinomycetes (Lockwood, 1959). Fungistasis has been decreased by addition of plant residues (Chinn & Ledingham, 1957), or organic compounds such as glucose (Dobbs & Hinson, 1953; Jackson, 1958*a*) to soil.

Many attempts have been made to elucidate the precise nature of soil fungistasis.

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On the basis of the similar inhibitory spectrum of various soils, Jackson (1958*b*) speculated that the inhibitors are likely to be a simple mixture of a few components, probably of microbial origin. Some workers have reported unidentified fungistatic substances in soil extracts and diffusates (Dobbs & Hinson, 1953; Hessayon, 1953; Jefferys & Hemming, 1953; Stover, 1958). Except for extraction of fungitoxic lignin-like material from soil (Lingappa & Lockwood, 1960) these results have usually been inconclusive, and the factors responsible for fungitoxicity have not been isolated and identified.

Special kinds of soil fungitoxicity such as those of the highly calcareous Newborough sands (Dobbs & Bywater, 1959) and the very acid (pH 2.8) Charnwood forest soils (Jackson, 1958*b*) seem to differ from the general fungistasis expressed by almost all soils. There is need to explain the nature of the widespread soil fungistasis. In the present investigation, the methods used by previous workers were repeated, and work was extended to include additional controls and new experiments. Attempts to determine whether the results from indirect assay methods in common use might lead to erroneous interpretation of soil fungistasis were of particular interest.

METHODS

Conidia from 4- to 7-day-old cultures of *Glomerella cingulata*, *Fusarium oxysporum* f. *lycopersici* and *Penicillium frequentans* maintained on potato dextrose agar were used. Conidia of *G. cingulata* do not germinate well in distilled water, but do so on water agar, whereas those of *F. oxysporum* f. *lycopersici* and *P. frequentans* germinate readily in distilled water and on water agar. It was necessary to wet spores of the latter with a $1/10^5$ dilution of polyoxyethylene sorbitan monooleate (Tween 80) to obtain uniform dispersion. A *Streptomyces* sp. and a *Pseudomonas* sp. which produced wide antibiotic inhibition zones on agar media were grown in yeast extract agar (Difco) and nutrient agar (Difco), respectively. All agar media used in these experiments contained 2% (w/v) Bacto agar (Difco). This material contains, according to the manufacturer, about 0.4% total protein (calculated from total N), 0.04% lipids (ether extractable), and 3.86% mineral content (ash). Bacto peptone (Difco) was used in 0.05% (w/v) concentration unless specified otherwise.

The soils used were Conover loam, muck, and a hardwood forest soil, all at 50–60% of saturation, from East Lansing, Michigan, U.S.A. Talc, diatomaceous earth (Celite), kaolin, bentonite, decolorizing carbon (Norit A), cellulose filter paper, or fibre glass filter paper were sometimes used in place of soil.

Many of the methods used by previous workers for assaying soil fungistasis were repeated in this laboratory. These included: (a) cellophan packets or folds (Dobbs & co-workers, 1953, 1957, 1959); (b) double agar layer method (Lockwood, 1959); (c) buried slide coated with seeded agar (Chinn, 1953); (d) water or peptone agar disks (2 mm. thick; Jackson, 1958*a, b*); (e) a modification of the method of Stover (1958) in which a layer of seeded agar was poured over a sterilized cellophan dialysis membrane. All these methods were used in Petri dishes filled to a depth of $\frac{1}{4}$ in. with soil and with the surface smoothed. Dishes were sealed with rubber bands and incubated at 28°. Assay media, except for buried slides, were pre-incubated on soil for 4–8 hr. (7 days for double agar layer method) followed by application of spores, then by continued incubation for another 16 hr. before counting germination. Three

microscope fields in each of 3 plates and a total of about 300 spores for each treatment were counted.

Direct methods of observing spore germination in soil were also used. About 50,000 conidia of the test fungi in 0.2–0.4 ml. of water were placed in a $\frac{1}{4}$ in. diameter area on moist soil in Petri dishes. Samples of these spores were removed after 16 hr. at 28° by lightly pressing 7 mm. diameter water agar disks on the demarked area. Disks and adhering spores were inverted on a glass slide for observation. In another method microscope slides containing a suspension of conidia of *Glomerella cingulata* were air dried to fix the spores to the glass surface, then were buried in soil and treated like those of Chinn (1953). In an extract method $\frac{1}{2}$ –1 million conidia of *G. cingulata* were mixed with 1 g. soil. After incubation for 16 hr. the soil samples were shaken with 10 ml. of 50 % (v/v) glycerol in water containing 0.5 % household detergent (Tide), and centrifuged for 2–20 min. at 1200 rev./min. (160 g); 70–80 % of the spores remained in the supernatant fluid which was free from all but colloidal soil particles. Spores were counted with a haemocytometer. Only ungerminated spores were recovered by this method. By comparison of the recovery from natural and autoclaved soils with controls refrigerated to prevent germination, estimates of germination could be calculated.

Several types of soil extracts were made. (1) Samples (100 g.) of loam or muck were extracted twice by shaking with 200 ml. of various C.P. grade solvents. (2) Drainage water from a tilled agricultural field of Conover loam of about 40 acres was collected from man-holes during dry weather when seepage was slow, and also after heavy rainfall; 16 l. samples were collected and passed through coarse filter paper. (3) Nearly 1000 lb. (about 450 kg.; 8.5 cu.ft.) of Conover loam soil in a greenhouse bench was periodically irrigated with excess water and up to 8 l. of drainage water collected each time in a polyethylene sheet. (4) Loam soil to a depth of 1 in. was placed on a $\frac{1}{2}$ in. deep bed of Norit A in glass trays and irrigated for 24, 48 or 72 hr. The recovered Norit A was extracted with various organic solvents. Water extracts of soils, or drainage water from the greenhouse bench were also treated with Norit A which was subsequently recovered and extracted with organic solvents.

Water extracts larger in volume than 1 l. were evaporated to $\frac{1}{5}$ volume in 20 min. at 40°–50° in a Precision Evaporator (Precision Scientific Co., Chicago, Illinois, U.S.A.). The resultant extracts, water extracts less than 1 l., and those of organic solvents were evaporated to a small volume under vacuum at 28–30° in a flash evaporator and assayed in seeded agar by direct incorporation or in filter paper disks ($\frac{1}{2}$ in. diameter).

Measurements of pH value and redox potential were made with a Beckman Model H2 pH meter.

All experiments were in triplicate and were repeated at least once. Sterile methods were used. In the case of natural soil, experiments were limited to 18 hr. in length when sterility was a consideration, due to the contaminating effects of motile soil microfauna.

RESULTS

Comparison of assay methods

Soil fungistasis was demonstrated by various methods, both indirect and direct. Some of the methods and typical results are given in Table 1. In addition, the modified method of Stover (1958) and the buried slide method of Chinn (1953) also

showed fungistasis in soil. Muck soil and hardwood forest soil gave inhibition of germination similar to that of Conover loam. Loss of fungistasis in autoclaved soils was shown by all methods; in these treatments fungi overgrew the entire plates. Factors such as thickness of agar, and temperature and duration of incubation on soil influenced the intensity of soil fungistasis as measured by the agar methods. By standardizing these factors, all three soils expressed a similar spectrum of inhibition of the test fungi in the order: *Penicillium frequentans* > *Glomerella cingulata* > *Fusarium oxysporum* f. *lycopersici*; *P. frequentans* was most readily inhibited.

Table 1. *Fungistasis of Conover loam soil assayed by various methods*

Treatment	Soil	Mean % germination of conidia*		
		<i>G. cingulata</i>	<i>F. oxysporum</i>	<i>P. frequentans</i>
Directly on soil	Natural	0	0	0
	Autoclaved	100	100	100
Glycerol extraction	Natural	0	—	—
	Autoclaved	94	—	—
Double agar layer	Natural	12	26	0
	Autoclaved	90	95	92
Cellophan packets	Natural	0	0	0
	Autoclaved	100	100	100
Water agar disks	Natural	0	0	0
	Autoclaved	98	100	100
Buried slide	Natural	0	0	0
	Autoclaved	100	92	92
Water agar control		98	100	90

* Mean of nine microscope fields with a total of approximately 300 conidia.

Table 2. *Fungistatic effects of different soils and substrates*

Soil or substrate		Mean % germination of conidia of <i>G. cingulata</i> *	
		Direct†	Agar disk‡
Conover loam	Natural	0	6
	Autoclaved	100	100
Muck	Natural	0	12
	Autoclaved	96	100
Forest soil	Natural	0	0
	Autoclaved	96	95
Sand	Natural	4	16
	Autoclaved	40	100
Fibre glass filter paper	Autoclaved	0	90
Cellulose filter paper	Autoclaved	0	90
Charcoal (Norit)	Autoclaved	0	10
Bentonite	Autoclaved	6	98
Kaolin	Autoclaved	0	90
Celite	Autoclaved	0	90
Talc	Autoclaved	0	95

* Mean of nine microscope fields with a total of approximately 300 conidia. Results were similar with conidia of *P. frequentans* and *F. oxysporum* f. *lycopersici*.

† Spores placed directly on soil surface.

‡ Spores placed on agar disks after disks were preincubated for 4 hr. on soil or substrate.

Fungistatic effects of substrates other than soil

There are reports that substances such as kaolin, alumina, slate dust (Dobbs & Hinson, 1953) and a calcareous sand (Dobbs & Bywater, 1959) decrease germination of fungus spores. Autoclaved cellulose filter papers, washed fibre glass filter papers, activated charcoal, bentonite, kaolin, talc, sand and diatomaceous earth (Celite) were tested for fungistatic effects by placing spores directly on them, and by the agar disk method. Results were similar with all three test fungi. The results for *Glomerella cingulata* (Table 2) show that these materials were fungitoxic when autoclaved and assayed by direct methods, but the fungistatic substances were apparently not diffusible, with the exception of those from sand and charcoal. Since fungistasis by soils was removed by autoclaving and the fungistatic substances were diffusible, it seems unlikely that the widespread soil fungistasis is due to clays or other colloidal or mineral materials.

Soil extracts

From experimental results similar to those in Table 1, previous workers have suspected the existence of diffusible fungistatic substances in natural soil. Following their lead, 100 g. samples of Conover loam soil were extracted with 50, 70 and 95 % (v/v) ethanol in water, *n*-butanol, methanol, acetone, chloroform, chloroform + methanol (1+1 by vol.), light petroleum ether, ethyl ether or water. When the concentrated extracts were assayed in water agar no inhibition of spore germination was observed; instead, stimulation of germination and growth was often observed. Likewise, concentrated drainage water from 8.5 cu.ft. of soil in the greenhouse, and from 40 acres of field soil did not inhibit germination. However, a more elaborate extraction of washed and defatted muck or mineral soil with 95 % (v/v) dioxane or ethanol in water yielded a fungistatic extract in the lignin-like fraction (Lingappa & Lockwood, 1960). This work will be discussed elsewhere (Lingappa & Lockwood, to be published).

The presence of stimulatory substances in soil extracts suggested that the expression of fungistatic substances might be masked. Concentrated extracts were chromatographed on filter paper strips in an attempt to separate inhibitory from stimulatory fractions. Chromatograms were developed in ethanol + acetic acid + water (12+6+1 by vol.), and in *n*-butanol + acetic acid + water (4+1+5 by vol.) and assayed on water agar seeded with *Glomerella cingulata*. Localized areas of stimulation of growth but no inhibition of spore germination were detectable in the assay plates. Strips of filter paper were also inserted into moist soil and diffusible materials from the soil allowed to accumulate at the tips of the hanging wicks (Wilson, 1958). After 16 hr. the strips were sterilized by immersing the buried end in 70 % (v/v) ethanol in water until the solvent reached the tip. When assayed on seeded agar no inhibitory substances were shown; again, areas of growth stimulation were evident.

Tests for volatile fungitoxic substances

The production of volatile antibiotic substances by micro-organisms has been reported (Bilal, 1956), and Dobbs & Bywater (1957) suspected that in some of their soils volatile fungitoxic substances might be present. Therefore it appeared possible

that our failure to extract fungistatic substances from soil might be due to loss of such volatile materials during the evaporation of solvents. Experiments were designed to detect such substances in natural soil. A coverglass coated on one side with a thin layer of water agar was inverted over a Van Tieghem ring and sealed with Vaseline. The rings were pressed into moist soil so that only a few mm. of space was left between soil and agar surface. Agar so exposed for 12–18 hr. at 28° was streaked with spores of the three test fungi and incubated again over soil or in moist chambers. Exposure for longer periods was not considered desirable because of inevitable contamination. In other experiments agar seeded with *Glomerella cingulata* was exposed to the soil atmosphere in Petri dishes for 16 hr. No inhibition of spore germination was observed in any of these tests. Absence of volatile fungitoxic materials in soil is also suggested by observations of Dobbs & Hinson (1953) that conidia on buried slides had germinated in pockets of condensed water. Similar observations were made in the spaces of buried Nylon gauze (Waid & Woodman, 1957). It was also our observation that complete contact of cellophan or agar disks with soil was necessary to avoid islands of spore germination over the air pockets. For these reasons it is believed that natural soil fungistasis is not caused by volatile substances from soil.

pH value and redox potential

The well known effects of pH value and redox potential of the medium on spore germination and growth of micro-organisms suggested the examination of these factors in natural soil fungistasis, although there appears to be no evidence that the pH of soils functions in this regard (Dobbs & Hinson, 1953; Jackson, 1958*a*). Table 3

Table 3. *Relation of pH value and redox potential of soil to fungistasis*

Substrate	Redox potential (mv)*	pH* value	% germination†
Natural Conover loam	216	7.9	—
Autoclaved Conover loam	198	7.5	+
Natural muck	192	6.5	—
Autoclaved muck	176	7.2	+
Natural Conover loam + 0.5 % peptone	—120	7.6	—
Natural muck + 0.5 % peptone	—36	7.5	—
Natural Conover loam + 0.5 % sucrose	—420	6.2	—
Water agar	276	5.8	+
Water agar in contact with soil 1 day	276	7.5	—

* Measurements made 16 hr. after amendments added, at time of taking spore germination data.

† Assays were by direct addition of conidia of *G. cingulata*, *F. oxysporum* and *P. frequentans* to soil. + = 20–100 % germination; — = 0–19 % germination.

shows that addition of small amounts of nutrients to soil did not annul fungistasis although drastically lowering the redox potential, whereas autoclaving soils annulled fungistasis while decreasing redox potential only slightly. Water agar developed strong fungistasis when incubated in contact with soil for 24 hr., but the pH did not change to an unfavourable value and the redox potential did not change at all. The soils in all treatments were at pH values favourable for spore germination, yet such soils were fungitoxic. For these reasons the pH and redox conditions of normal soil do not appear to be the cause of soil fungistasis.

Annulment of fungistasis

Since various workers have reported a decrease in fungistasis when soil was treated with plant residues or glucose (Dobbs & Hinson, 1953; Chinn & Ledingham, 1957), or when glucose was added to soil extracts (Stover, 1958) or agar disks (Jackson, 1958*a*), it was of interest to know whether spores already inhibited could be stimulated to germinate by nutritional supplementation. It has been shown that inhibited spores were viable (Lockwood, 1959). Spores of test fungi which had lain dormant for 5 days on the inhibitory bottom layer of double agar layer plates were removed with uniform disks of agar and transferred to separate Petri dishes. Known amounts of chemical solutions (0.2 ml. of 0.01 M solutions) were placed on these disks. Of the chemicals used only L-cysteine HCl and L-methionine prevented germination of conidia in controls. Similar results were obtained with all three test fungi. Those for *Glomerella cingulata* (Table 4) show the great effectiveness of peptone, even in small amounts (0.05 %, w/v), in annulling fungistasis. Glucose failed to induce germination of inhibited spores under the conditions of these tests. Various amino and organic acids and water were largely ineffective in annulling fungistasis. Osmotic gradients created by solutions of glucose, sorbitol, mannitol or polyglycols of different molecular weight did not annul fungistasis nor did they interfere with spore germination in controls. Therefore lack of osmotic shock probably would not explain failure of fungal spores to germinate in soils.

Table 4. *Annulment of fungistasis of Glomerella cingulata conidia*

Chemical*	% germination†	Chemical*	% germination†
Glycine	0	Succinic acid	15
L-Histidine	0	Glucose (0.1 M)	0
L-Cystine	8	Peptone (0.5 %, w/v)	90
L-Cysteine HCl‡	0	Peptone (0.05 %, w/v)	84
L-Methionine‡	0	Mannitol	10
DL-Phenylalanine	0	Mannitol (0.1 M)	12
L-Tyrosine	0	Sorbitol	0
L-Aspartic acid	30	Polyglycols (200, 300, 400, 600 mol. wt.)	0
L-Glutamic acid	12	Water	0
p-Aminobenzoic acid	0	Untreated	0

* All are 0.01 M unless otherwise indicated; 0.2 ml. placed on 1.5 mm. thick × 8 mm. diam. water agar disks.

† Average of nine microscope fields with a total of 300 spores. The spores were inhibited for 5 days by the double agar layer method before reversal. Results were similar using conidia of *P. frequentans* and *F. oxysporum* f. *lycopersici*.

‡ Inhibited spore germination in controls.

Destruction of conidia in soil

The conidia of at least some fungi are destroyed rather rapidly in contact with soil, a fate not revealed by indirect methods of observations. Conidia of *Glomerella cingulata*, *Fusarium oxysporum* f. *lycopersici* and *Penicillium frequentans* were placed directly on soil. After 3, 6 and 9 days of incubation, direct microscopical observation revealed large numbers of empty or decomposing conidia of *G. cingulata* and *P. frequentans*. It was difficult to detect the microconidia of *F. oxysporum* f. *lycopersici* because of their small size. By plating these soils after 6 days, large numbers

of colonies of *P. frequentans*, a few of *F. oxysporum* f. *lycopersici*, and none of *G. cingulata* developed. After 9 days the number of colonies of *P. frequentans* was decreased and none of *F. oxysporum* f. *lycopersici* developed. No detectable growth of micro-organisms could be seen on the conidia of these fungi. In autoclaved soil the fungi had overgrown the plates as profusely sporulating mycelia. Lysis of spores by soil was also observed by Subramanian (1946, 1950) and by Park (1955).

Source of fungistasis as assayed by indirect methods

When agar methods were used for assaying soil fungistasis, inhibition occurred more rapidly when 0.05 % (w/v) or more peptone was present than with water agar alone. The bottom layer of double agar layer plates became inhibitory in 3 days when the top agar layer contained 0.5 % (w/v) peptone, but without peptone 7–8 days were required. Similarly, peptone agar disks became fungistatic in 4 hr. when pre-incubated on soil at 28°, but 8 hr. were required for water agar disks to become similarly fungistatic. At 21° water agar disks pre-incubated for 4 hr. gave 60 % germination of conidia of *Fusarium oxysporum* f. *lycopersici*, 20 % of *Glomerella cingulata*, and none of *Penicillium frequentans*. Peptone agar disks similarly treated caused complete inhibition of all three fungi.

Table 5. *Effect of pre-incubation temperature on the expression of soil fungistasis by indirect methods*

Method†	Pre-incubation time	Substrate	Pre-incubation temperature	
			1°	28°
Double (water) agar layer	5 days	Natural soil	96	6
	14 days	Natural soil	92	0
	14 days	Autoclaved soil	98	96
Water agar disks on cellophane	8 hr.	Natural soil	98	0
	16 hr.	Natural soil	96	0
	48 hr.	Natural soil	92	0
	48 hr.	Autoclaved soil	92	96
Water agar disks	24 hr.	Fungistatic agar layer	0	0

* Average of nine microscopic fields with a total of 300 spores.

† Agar disks or layers were pre-incubated on Conover loam soil or fungistatic agar layer for various time intervals, then streaked with conidia of *G. cingulata* and incubated for 16 hr. at 28°.

These observations raised the question as to whether fungistasis was caused by substances present in soil, or whether fungistatic substances were generated on the surface of the assay medium and then diffused into it during incubation. Since very little or no growth of micro-organisms takes place at 1°, pre-incubation of assay media at this temperature was tested to investigate this point. The double agar (water) layer and agar (water) disk methods were used, and a layer of sterilized cellophan was interposed between soil and agar. Table 5 shows that the agar media were not toxic to spore germination by *Glomerella cingulata* when pre-incubated for extended periods of time on soil at 1°, then removed and incubated with fungal spores at 28°. The soil did not lose the capacity to express fungistasis by exposure to

cold temperatures, since subsequent pre-incubation of new agar disks on the same soils at 28° caused inhibition of spores subsequently applied to the disks. To test the possibility that fungistatic substances might not diffuse at 1°, double agar layer plates were pre-incubated with soil for 12 days at 28°, thus making the bottom layer fungistatic. The fungistatic layers were then covered with sterilized cellophan. Water agar disks were placed on the cellophan and incubated at 1° for 24 hr. the disks then being removed and placed on water agar. Spores of test fungi applied to these disks and incubated at 28° for 16 hr. did not germinate (Table 5). This indicated that fungistatic substances which had accumulated in agar were indeed able to diffuse into assay disks at 1°. Results were the same with all three fungi, strongly indicating that fungistasis as assayed by the indirect methods (agar or cellophan) was due to production of fungistatic substances by soil microbes growing on the contact surface of assay medium and soil, rather than to the presence of a reservoir of such substances in soil.

Table 6. *The effect of amount of soil on expression of fungistasis by indirect methods*

Substrate or method*	Thickness of soil	% germination of <i>G. cingulata</i> conidia†
Double (water) agar layer	1 cm.	6
	1 mm.	8
(Water) agar disks	6 cm.	2
(Water) agar disks	< 0.1 mm.	12
(Peptone) agar disks	< 0.1 mm.	0
Cellophan	1 cm.	0
Cellophan	< 0.1 mm.	0
Mixed soil organisms assayed with water agar disks‡	0	0
Natural soil-direct	1 cm.	0
Autoclaved soil-direct	1 cm.	88

* Pre-incubation was 6 hr. except for double agar layer which was 7 days.

† Average of nine microscope fields with a total of 300 spores.

‡ Diluted soil suspension was mixed with water agar or peptone agar, incubated 3 days, and assayed with water agar disks.

This possibility was examined further by using different amounts of soil in contact with assay media. Fungistasis of *Glomerella cingulata* conidia (Table 6) was expressed equally by agar disks incubated on a soil layer < 0.1 mm. thick as on a soil layer 60 mm. thick. Bottom layers of double agar layer plates incubated for 7 days at 28° became equally fungistatic whether 16 g. soil, 10 mg. soil, or a thin layer of a 1/1000 dilution of soil in water was placed on the top layer. The amount of soil did not affect the time required for the bottom layer to become fungistatic. Furthermore, 1/1000 dilutions of soil incubated for 3 days on peptone or water agar, then assayed with agar disks or cellophan strips gave the usual fungistatic effects. Similarly, mixed cultures of soil microbes isolated from soil dilution plates and grown for 3 days on peptone agar caused water agar disks placed on them to become fungistatic. Peptone agar seeded with an antagonistic *Streptomyces* sp. or *Pseudo-*

monas sp. also produced fungistasis in agar disks. Inhibition, as compared with *G. cingulata*, was somewhat greater with *Penicillium frequentans*, and slightly less with *Fusarium oxysporum* f. *lycopersici*, in these tests.

The possible production of fungistatic substances on the assay medium surface was further tested by incorporating antibacterial antibiotics into agar disks, pre-incubating them on soil for 2 hr., then removing them to moist fibre glass filter papers which were soaked with the antibiotics, and assaying the generation of fungistasis in these disks. Results were similar with all three test fungi. Those for *Glomerella cingulata* (Table 7) indicated a decrease in soil fungistasis by these methods. The presence of antibiotic-resistant organisms in soil no doubt prevented complete suppression of fungistasis. These results support the view that soil fungistasis as assayed by indirect methods is due to growth of micro-organisms on the assay medium.

Table 7. *Effect of antibacterial antibiotics on soil fungistasis as assayed with peptone or water agar disks**

Antibiotic	% germination of <i>G. cingulata</i> conidia		
	Water agar disks	Peptone agar disks	Water agar disks (no soil)
K Penicillin G	40	90	98
Streptomycin sulphate	90	20	95
Neomycin	67	25	100
Vancomycin	70	90	92
Chloramphenicol	30	75	96
Control (no antibiotic)	12	0	—

* Agar disks containing antibiotics (1 mg./ml.) were pre-incubated directly on soil for 2 hr., then transferred to fibre glass filter paper moistened with the same antibiotic.

The above results were further supported by microscopical observation of the surface of assay media. Water agar or peptone agar disks, or sterile cellophan strips were placed on a smooth moist soil surface and pressed so as to make complete contact. After 8, 12, 24 and 48 hr. they were carefully lifted, inverted on a microscope slide, stained with phenol+rose bengal and washed gently in water. Such preparations revealed extensive colonization of the surface in contact with soil by bacteria within 8 hr. (Pl. 1, figs. 1, 2), and colonization by actinomycetes in 24 hr. (Pl. 1, figs. 3, 4). In this connexion, water agar has been found to be a good substrate for development of soil actinomycetes (Y. Lingappa & Lockwood, 1961).

Antibiotic and nutritional substances from fungal spores and their possible relation to fungistasis in soil

Freshly collected teliospores of the maize smut fungus *Ustilago zeae* did not germinate when placed directly on natural loam soil, but 40 % germinated on water agar in 16 hr. Germination and production of aerial sporidia occurred on autoclaved soil. Samples (5 g.) of air-dry smut teliospores were washed twice by shaking for 10 min. at a time with 100 ml. 50 % (v/v) ethanol in water. Another 5 g. sample was

spread on the surface of a layer of 20 g. natural soil, moistened, and incubated for 4 days at 24°, after which the spores were scraped off and extracted similarly. When the washings from the air-dry spores were evaporated on filter paper disks and assayed by the agar diffusion method against the organisms arising from a diluted soil suspension, they showed a strong antibiotic zone against these organisms as well as against *Fusarium oxysporum* f. *lycopersici*, *Penicillium frequentans*, *Glomerella cingulata*, *Pseudomonas* sp., and the *Streptomyces* sp. A very wide zone of stimulation occurred outside the zone of inhibition. On the other hand, washings from spores incubated in soil for 4 days showed no zone of inhibition, and only a slight zone of stimulation, suggesting exhaustion or biological breakdown of these materials. On the basis of these scanty observations with one fungus, the possibility is suggested that individual spores might behave as microsubstrates in the soil. Nutrients released by fungus spores could stimulate growth of antagonistic microflora in the vicinity of the spore, preventing its germination. The release of antibiotics from the spore could prevent or delay colonization of the actual spore surface.

DISCUSSION

In spite of numerous attempts to obtain fungistatic substances from soil, a characterized fungistatic principle has not been isolated with the exception of the lignin-like preparation of Lingappa & Lockwood (1960). In the work reported in the present paper, soil extracts were stimulatory to the growth of fungi. In spite of the failure to extract fungistatic substances, the fact that soil extracts are often stimulatory, as reported here and by others (Dobbs & Hinson, 1953; James, 1958; Park, 1956*b*), and the fact that spores of fungi which germinate readily in water are inhibited by soil (Dobbs & Hinson, 1953) show clearly that soil fungistasis cannot be the result of insufficient nutrients in soil.

A possible group of suspected fungistatic substances in soil includes the antibiotics. On the basis of researches to date (Brian, 1957), the production and accumulation of detectable quantities of antibiotics from natural unamended and uninoculated soil is considered to be unlikely. The present workers carried out very extensive and intensive extractions of natural soils and failed to demonstrate any antifungal antibiotics. While the accumulation of antibiotics in natural soil has not been demonstrated, evidence generally favours the production of antibiotics on fresh organic substrates in micro-environments of the soil (Brian, 1957). As the amounts produced are likely to be extremely small, when the bulk of the soil is considered, their demonstration has been by necessity indirect. The addition of a fresh organic substrate, such as agar or cellophan, might supply a source of nutrients and support microbial growth and antibiotic production. Such a possibility is supported by the short generation time of many bacteria and the very large number of micro-organisms in soil. An average soil containing 10 million individual micro-organisms/g. would provide an initial and enormous inoculum of up to 100,000 microbes in contact with a 1 cm. square agar block placed on it. Present information (Brian, 1957) permits us to assume that 40–50 % of the soil micro-organisms produce antibiotics. The present work and that of others (Y. Lingappa & Lockwood, 1961) shows that antibiotics are produced by actinomycetes growing on deficient media such as water agar, and that fungistatic substances are produced as well from mixed cultures as from single cultures of antibiotic-producing micro-organisms.

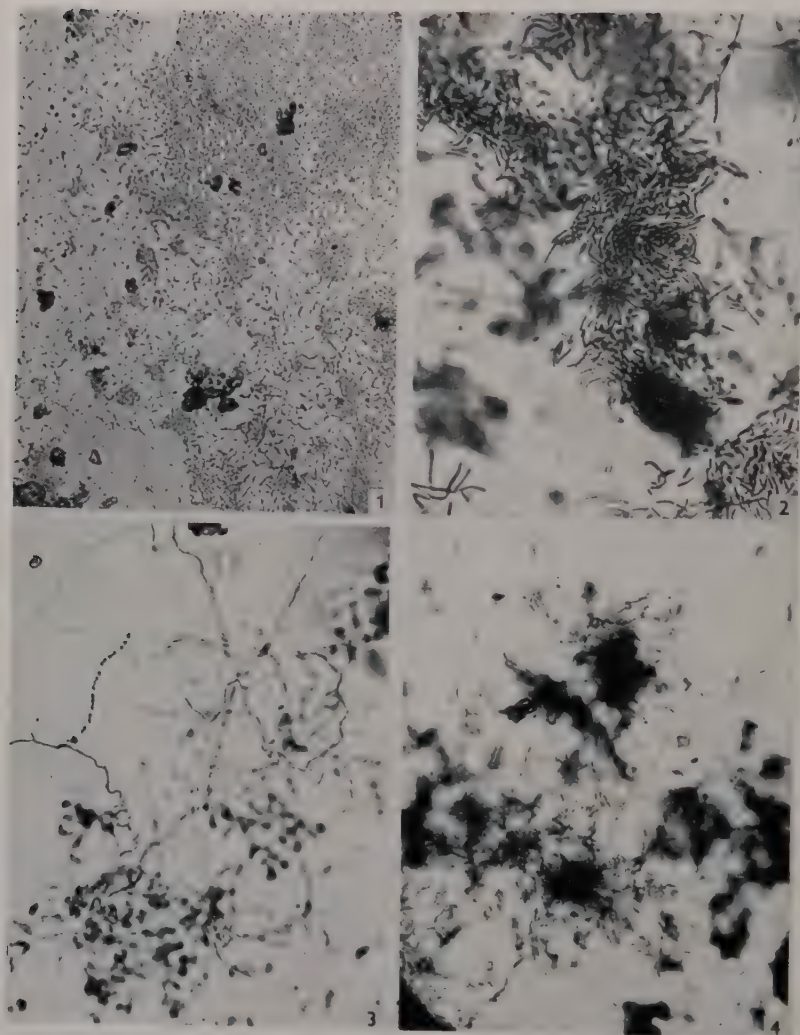
This explanation of the inhibition of fungal spore germination on assay media in contact with soil was supported by five lines of evidence. (1) Peptone agar became fungistatic more rapidly than water agar in contact with natural loam soil. (2) When agar media were pre-incubated on soil at 1°, a temperature which permits diffusion of fungistatic substances but largely prevents microbial growth, no fungistasis occurred. (3) The same degree of fungistasis was obtained with agar disks pre-incubated with thick layers of soil, minute amounts of soil, mixed cultures of soil microbes, or pure cultures of *Streptomyces* sp. or *Pseudomonas* sp. (4) Decrease of fungistasis was observed when high concentrations of antibacterial antibiotics were added to agar disks used for assaying soil. (5) Microscopic observations showed dense growth of micro-organisms within short periods on the contact surfaces of agar and cellophan with soil.

These indirect techniques, then, do not demonstrate the presence of fungistatic substances in soil, but provide a substrate for growth of micro-organisms which produce antibiotic substances, thus rendering the assay media fungistatic. Nevertheless, failure of fungal spores to germinate in natural soil is indeed a fact as shown by direct methods of observation. Understanding of the action of the indirect assay methods may provide some insight about the mechanism of inhibition involved. The introduction of spores into soil can create, qualitatively, as much a nutrient substrate as a piece of water agar, cellophan or straw. The experiments with smut spores showed that these liberated diffusible substances which stimulated growth of a variety of micro-organisms. If speculation is extended to individual spores in soil one might expect, as a result of nutrients diffusing from the spores, stimulation of surrounding microflora and resultant inhibition of fungal spore germination. An analogous situation is the reported stimulation of vegetative growth of the fungus *Fusarium oxysporum* f. *lycopersici* and the production of the antibiotic fusaric acid in the vicinity of the root surface of tomato plants growing in unsterilized soil or sand (Kalyanasundaram, 1958). Observations of various workers (Subramanian, 1946, 1950; Park, 1955; Chinn, 1953; Lockwood, 1959) and the present work showing that fungus mycelium and conidia are destroyed ultimately by soil, and reports of the colonization of the surface of fungal structures in soil (Mitchell, Hooton & Clark, 1941; Subramanian, 1946, 1950; Waid & Woodman, 1957; Stover, 1958) support the assumption that growth of soil micro-organisms on or in the vicinity of fungal structures is involved in the over-all fungitoxicity. Lysis of fungi appears to be the end result of extended microbial activities in the vicinity of spores and mycelium in soil.

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EXPLANATION OF PLATE 1

Fig. 1. Bacteria colonizing water agar disks after incubation on moist Conover loam soil for 8 hr. at 28°. ×400.

Fig. 2. Bacteria colonizing cellophan after incubation on moist Conover loam soil for 8 hr. at 28°. ×800.

Fig. 3. Actinomycetes colonizing water agar disks after incubation on moist Conover loam soil for 24 hr. at 28°. ×800.

Fig. 4. Actinomycetes colonizing water agar disks after incubation on moist Conover soil for 24 hr. at 28°. ×400.

Physiology of the Conjugation Process in the Yeast *Hansenula wingei*

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SUMMARY

The yeast *Hansenula wingei* is a favourable organism for study of the physiology of the conjugation process (cell fusion). Microscopic observations on fusion are presented which reveal that the mating cells in contact fuse by a softening of the cell wall, followed by formation of a conjugation tube, dissolution of the cross-walls between them, and formation of a new bud at the point of juncture of the two cells. A simple technique for studying fusion in a liquid medium is described. Up to 80 % of the cells will fuse in 5 hr. at 30° in a medium containing an energy source, MgSO₄ and potassium phosphate, under conditions in which no growth or budding of unmated cells would occur. Synthesis of new protein is required for fusion as shown by inhibition by amino acid analogues. The precursors for this new protein come from the amino acid pool. Both mating types must be able to function for conjugation to occur. It is postulated that each mating type produces an inducer which diffuses into the opposite type. Each inducer brings about the synthesis of a wall-softening enzyme which acts upon the cell producing it. Cell fusion is viewed as an extension of the normal budding process.

INTRODUCTION

The physiology of reproduction in fungi was reviewed by Hawker in 1957. It is clear from this review how meagre is knowledge of the biochemical processes involved in reproduction. In particular, there has been no analysis of the physiological processes involved in cell fusion, the first step in the mating cycle in fungi and most other organisms. As Hawker emphasized, the higher fungi are not suitable for such an analysis, partly because they are difficult to handle and partly because differentiation of cells into various types, some involved in mating and some not, makes an analysis at the cellular level difficult.

The yeasts would seem to be more favourable material for such studies. In a sexually reproducing yeast each cell may function either in vegetative growth or in mating. In the heterothallic yeasts, two mating types occur, genetically determined, which can be kept in culture indefinitely as haploid strains. Mating can then be induced at will by mixing the two mating types.

The only earlier work on mating in yeast is that of Nickerson & Thimann (1941, 1943). The present work is an outgrowth of studies on the nature of the mating reactions in *Hansenula wingei*. This yeast, isolated by Wickerham (1956), is unusual

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in that cells of the two mating types exhibit an extremely strong attraction for each other, so that when mixed in mass culture, a marked agglutination occurs. Analysis of the nature of the attractive forces (Brock, 1959*a, b*) showed that one mating type, strain 5, possesses a specific carbohydrate on its cell wall, whereas the other mating type, strain 21, possesses a specific protein. Mating agglutination is due to a combination between these complementary macromolecules, analogous to a reaction between an antibody and antigen. Because of the strong attraction between the mating types, cells can be mixed and remain in contact throughout extensive aeration and other physiological manipulation, and during this time cell fusion occurs. It has thus been possible to examine some of the factors involved in the fusion process. Preliminary accounts of this work have been given (Brock, 1959*c, d*).

METHODS

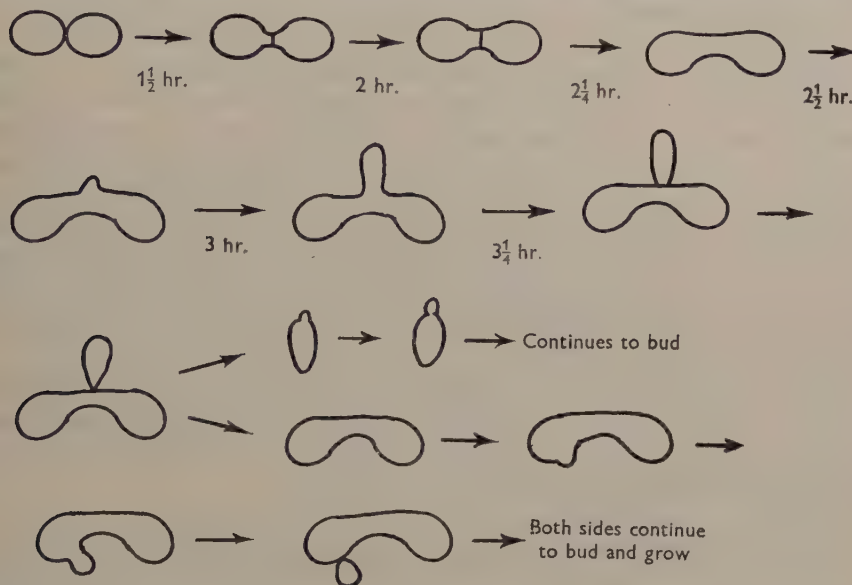
Strains used. The yeasts used in this study were supplied by Dr L. J. Wickerham, U.S. Department of Agriculture, Peoria, Illinois, U.S.A. They were *Hansenula wingei* NRRL Y-2340, strains 5 and 21, which were the agglutinative mating types.

Growth of cells. The cells were grown in liquid medium of the following composition: glucose 30 g., yeast extract Difco (Detroit, Michigan, U.S.A.) 7 g., KH_2PO_4 5 g., in 1000 ml. distilled water. This medium was dispensed in 100 ml. amounts into 500 ml. Erlenmeyer flasks, autoclaved 15 min., 121° , and inoculated with cells from stock agar slopes. The flasks were then placed on a reciprocating shaker and incubated for 18 hr. at 30° . The cells were harvested by centrifugation, washed twice with distilled water and suspended in distilled water at the same cell concentration as in the growth medium.

Cell fusion assay. Mating was carried out by mixing together 5 ml. amounts of suspensions of each cell type with 0.1 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and centrifuging for 5 min. The packed cells became intimately mixed and agglutinated strongly. The supernatant fluid was then decanted and the packed cells resuspended in 10 ml. of conjugation medium (glucose, 1 %, w/v; potassium phosphate (pH 5.7); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 %, w/v). Although the cells resuspended poorly because of the agglutination, the mixture was made as homogeneous as possible. This suspension was then poured into a 1 in. diameter shell glass vial which was then placed on a reciprocating shaker at 150 rev./min. Usually the vials were shaken for 5 hr. at 30° . At this time the suspension, which was still quite agglutinable, was decanted into a centrifuge tube and sedimented. The supernatant fluid was discarded and the cells suspended in 10 ml. 8M-urea. The suspension was placed in an autoclave for 10–15 min. at 120° to deagglutinate the cells, the 8M-urea serving to keep the cells from re-agglutinating when they cooled down. Although this treatment effectively converted the clumped cells to a fine suspension, the conjugated cells remained together when fusion had occurred. It was then possible to take small samples of these cells, stain them with dilute crystal violet, and examine them under oil immersion at $\times 950$ magnification. The number of conjugants and the total number of cells were counted in a number of microscope fields. From these data the % conjugation was calculated by the method of Nickerson & Thimann (1943), counting each conjugating pair as two cells and each single cell as one cell. The data presented here report the

RESULTS

Cells of each strain grown for 24 hr. in glucose yeast-extract KH_2PO_4 broth on the shaker were mixed in equal parts in the same medium containing 20 % (w/v) gelatin, the mixture placed on a coverslip on a hanging-drop slide, ringed with paraffin wax and observed under a Leitz phase microscope at room temperature. At the cell concentrations used about one-half of the cells were single, and the other half occurred



in pairs touching each other. The single cells budded but never exhibited any attraction for adjacent cells. Pairs of cells in contact were observed over a period of 4-5 hr. for conjugation behaviour. The results of the observations are shown in the diagram (Fig. 1). In the first 1-1½ hr. the cells in contact showed no visible behaviour. By 2 hr. many of the paired cells showed conjugation tubes. These tubes were observed to form by an extension of the portion of each cell at the point where it touched its mate. It should be emphasized that at no time were conjugation tubes observed to be formed by growth together of two separated cells, but always by an extension of each of the two cells in contact. In effect, the formation of the conjugation tube was due to each cell pushing its main body away from its mate. The conjugation tube appeared to result from a softening of the cell wall. In *Saccharomyces cerevisiae* Levi (1956) observed conjugation processes between cells not in contact, implying that some extracellular substance induced the process. There was no evidence for such a situation with the organism used in the present work.

At first the cross-walls between the two cells remained intact. At about 2 hr. the cross-walls disappeared. This occurred during a short period of time and was often missed. In one case the cross-walls disappeared within a 10 min. period.

Shortly after the cross-walls disappeared, a small bud formed at the mid-point of the conjugation tube and at a right angle to it. It is assumed that the bud formed here because this was the softest part of the wall. This small bud grew rapidly and by 3 hr. appeared to be fully grown. Shortly after 3 hr. a wall between this bud and the parent cells was quickly formed, often being complete in 5–10 min. This new bud always seemed longer than either of the parent cells, often appearing moderately filamentous. After enlarging fully this first bud could then form new buds, usually from the tip. Later, this bud on the conjugation tube might become completely detached from the parents.

Later, the parent cells often budded individually from locations far removed from the conjugation tube. These buds were morphologically similar to the parents, rather than elongated like the conjugation tube bud. Nothing is known about the nuclear phenomena in the mating process, and no bodies which could definitely be called nuclei were seen. It is assumed that after the cross-wall in the conjugation tube dissolves, nuclei from each parent can move into the tube and either fuse or remain separate. In either case there must be nuclear migration into the bud which forms off the conjugation tube, and this new bud must acquire nuclear material from both parents. Preliminary genetic evidence (Herman, 1959) suggests that the hybrids from matings possess characteristics of both strains but may be heterocaryons instead of true diploids.

In the physiological studies which follow, the only process under consideration is cell fusion and the formation of conjugation tubes. Later stages of growth of the mated cells are not considered and probably do not occur, since the medium used in most studies is inadequate for growth.

Physiology of conjugation

A preliminary experiment was made to determine the time course of conjugation under the conditions selected. In this experiment the conjugation medium was the growth medium. As shown in Table 1, conjugation began after about 1½ hr. and reached about 50 % after 4–5 hr. In later experiments with other conjugation media the % conjugation was somewhat higher. A time of 5 hr. was selected for all subsequent experiments.

Table 1. *Time course of conjugation in Hansenula wingei*

Cells of strains 5 and 21 were mixed and suspended in conjugation medium (glucose + yeast extract + phosphate) and shaken for various times at 30°; samples removed and assayed microscopically for % conjugants as described in Methods.

Time (hr.)	Conjugants (%)	Time (hr.)	Conjugants (%)
0	0	2.5	40
0.5	0	3.0	47
1.0	0	4.0	47
1.5	8.5	6.0	57
2.0	32		

The effect of various nutrient factors on conjugation was studied next, and the results are in Table 2. In all cases except with water the cells remained firmly clumped throughout the incubation period on the shaker; the cells in water, however, no longer remained agglutinated. This is in line with previous observations that cations are necessary for agglutination. Therefore, in all subsequent experiments 0.1 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was present in the conjugation medium. Further data in Table 2 indicate that no conjugation occurred unless glucose was present; neither yeast-extract nor casein hydrolysate replaced glucose. An added nitrogen source was not necessary for conjugation.

Table 2. *Nutrient requirements for conjugation in Hansenula wingei*

Cells of strains 5 and 21 were mixed and suspended in the media indicated, shaken 5 hr. at 30°, then assayed microscopically for % conjugation as described in Methods.

Composition of conjugation medium	Conjugants at 5 hr. (%)
Water	<1
0.1 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	<1
0.05 M-phosphate (pH 5.7) + 0.1 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	2
1.0 % (w/v) glucose + 0.05 M phosphate + 0.1 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	55
1.0 % yeast extract + 0.05 M phosphate + 0.1 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	13
1.0 % casein hydrolysate + 0.05 M phosphate + 0.1 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	10
0.5 % NH_4NO_3 + 0.05 M phosphate + 0.1 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	4
1.0 % glucose + 0.5 % (w/v) NH_4NO_3 + 0.05 M phosphate + 0.1 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	58
1.0 % glucose + 1.0 % casein hydrolysate + 0.05 M phosphate + 0.1 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	51
3 % (w/v) glucose + 0.7 % (w/v) yeast extract + 0.5 % (w/v) KH_2PO_4 (growth medium)	54

Table 3. *Effect of energy source on conjugation in Hansenula wingei*

Cells of strains 5 and 21 were mixed and suspended in conjugation media containing 0.05 M phosphate buffer (pH 5.7) + 0.1 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ + energy source as indicated, then shaken 5 hr. at 30° and assayed microscopically for % conjugants as described in Methods.

Energy source (%, w/v)	Conjugation at 5 hr. (%)	Energy source (%, w/v)	Conjugation at 5 hr. (%)
Glucose, 1.0	69	Sucrose, 0.5	40
Glycerol, 2.0	51	Lactose, 0.5	2
Ethanol, 4.0	17	Trehalose, 0.5	15
Mannose, 1.0	50	Na citrate, 1	0
Galactose, 1.0	4		

A study of energy sources for conjugation was carried out with the results shown in Table 3. Only substances which had been reported by Wickerham (1956) to be utilized for growth by this organism were useful as energy sources for conjugation. In all subsequent experiments the conjugation medium used was: 1.0 % (w/v) glucose, 0.1 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 M-potassium phosphate buffer (pH 5.7).

No growth occurred in this simple medium as shown by the following experiment. Cells of each strain were suspended separately in the conjugation medium and incubated for 5 hr., then samples removed and examined under the microscope. The number of single cells, budding cells, unequal pairs and equal pairs were counted (Campbell, 1957) to indicate whether growth had occurred; the total count was also

determined. The results after 5 hr. were compared with results obtained with unincubated cells. Table 4 shows that there was neither an increase in total numbers of cells nor an increase in number of budding cells. The number of buds and pairs decreased, while the number of single cells increased. The increase in number of single cells indicates that cell separation was the main process which occurred in this non-growth medium.

Table 4. *Budding and growth of cells of Hansenula wingei during 5 hr. incubation in conjugation medium*

Cells not mated, but suspended separately in conjugation medium (glucose + MgSO_4 + phosphate), shaken for 5 hr. at 30° , then examined microscopically and buds enumerated.

	Strain 5		Strain 21	
	0 hr.	5 hr.	0 hr.	5 hr.
Total count $\times 10^{-8}$ ml.	6.8	6.6	4.1	5.0
Single cells (%)	67	82	59	75
Budding cells (%)	12	4	10	9
Unequal pairs (%)	14	7	13	8
Equal pairs (%)	7	7	18	8

Both cell types must function in the conjugation process; this was shown in several ways. The antibiotic cycloheximide (Actidione; The Upjohn Co., Kalamazoo, Michigan, U.S.A.) completely inhibited conjugation at concentrations which inhibited growth. The wild-type cells were completely inhibited by cycloheximide $0.5 \mu\text{g./ml.}$ Antibiotic-resistant mutants were isolated from both mating types by periodic transfer to agar containing increasing concentrations of cycloheximide until mutants resistant to $100 \mu\text{g./ml.}$ were obtained. (These strains were isolated by Miss Alberta Herman and Mr D. Hunt.) The resistant mutants agglutinated and conjugated normally, both with each other and with wild-type cells. Table 5 shows the results when these mutants were mated with wild types in all combinations, with and without cycloheximide. Conjugation occurred in the presence of cycloheximide only when both strains were resistant, indicating that both strains must function for conjugation to occur.

Table 5. *Conjugation of cycloheximide-resistant mutants and wild-type cells of Hansenula wingei with and without antibiotic*

Cells of the two strains were mixed and suspended in conjugation medium (glucose + MgSO_4 + phosphate), shaken for 5 hr. at 30° , then % conjugants determined microscopically as described in Methods.

Strain 5	Strain 21	% Conjugation	
		No antibiotic	Cycloheximide $1 \mu\text{g./ml.}$
Wild	Wild	72	0
Wild	Resistant	57	0
Resistant	Wild	59	0
Resistant	Resistant	63	71

This same conclusion can be drawn from the action of ultraviolet (u.v.) radiation on the conjugation process. Washed cells of both strains were irradiated under a General Electric germicidal lamp at a distance of 15 cm. The cell suspensions were

5 ml. amounts in flat-bottom Petri dishes and were agitated continually during irradiation. These irradiated samples were then mated with unirradiated cells of the opposite type and conjugation allowed to occur. All operations were carried out in subdued light or in darkness to avoid photo-reactivation. As can be seen from Table 6, conjugation was quickly affected by short exposures to u.v. radiation. Viable counts of these irradiated cells indicated that ability to form colonies decreased at roughly the same rate as did ability to conjugate. These results also indicate that both types must function in the conjugation process.

Table 6. *Conjugation of ultraviolet-irradiated cells with unirradiated cells of opposite type of Hansenula wingei*

Cells of each strain were irradiated separately, then mixed with unirradiated cells of opposite mating type, shaken for 5 hr. at 30° in conjugation medium (glucose + MgSO_4 + phosphate), then assayed microscopically for % conjugants as described in Methods.

Time of exposure to u.v. radiation (sec.)	% Conjugation	
	Strain 5 irradiated	Strain 21 irradiated
0	68	68
5	56	52
10	38	45
20	21	33
30	20	19
60	2	2

The action of a number of metabolic inhibitors is presented in Table 7, showing that the usual metabolic inhibitors prevented conjugation.

Table 7. *Inhibition of conjugation in Hansenula wingei by metabolic inhibitors*

Cells of two mating types were mixed and suspended in conjugation medium (glucose + MgSO_4 + phosphate) containing various additions as indicated, shaken 5 hr. at 30°, then assayed microscopically for % conjugants as described in Methods.

Treatment	% Conjugation at 5 hr.	Treatment	% Conjugation at 5 hr.
Control	76	Dinitrophenol, 0.001 M	4
KCN, 0.05 M	4	Na azide, 0.001 M	0
Na arsenate, 0.001 M	63	Boric acid, 0.025 M	0
Na arsenite, 0.001 M	0	Nystatin, 10 $\mu\text{g.}/\text{ml.}$	0

Because of the marked morphological changes which occur during conjugation, it seems reasonable to assume that in each cell type there is the synthesis of an enzyme(s) which softens and eventually dissolves the wall between the cells. If enzymes are synthesized for this process, they might be inducible and might be synthesized *de novo* from constituents of the cell. It was shown by Spiegelman & Halvorson (1953) that induced enzyme synthesis in *Saccharomyces* makes use of the free amino acid pool of the cells for the materials needed for protein synthesis. Several experiments were therefore made to see whether the free amino acid pool is necessary for conjugation in *Hansenula wingei*. Although no quantitative determinations were made, this organism was found to possess an extensive amino acid pool which was extractable by hot water and which reacted with ninhydrin. This pool

was diminished effectively by starvation of the cells by aeration in 1 % (w/v) glucose + 0.05 M-phosphate buffer (pH 5.7). After aeration for 4 hr. there was no more ninhydrin-reacting material extractable by hot water remaining in the cells. Table 8 shows qualitatively the amino acid pool of these starved cells and the ability of starved cells to conjugate. These results show that starvation to decrease the free amino acid pool also decreased the number of conjugants, although the pool seemed to diminish faster than did the ability to conjugate. Spiegelman & Halvorson (1953) showed that a considerable degree of induced enzyme synthesis occurred even after there was no detectable amino acid pool.

Table 8. *Conjugation and amino acid pools of starved cells of Hansenula wingei*

Cells starved in glucose + phosphate for various times as indicated. Samples removed for extraction of amino acid pools with hot water, and amount of pool determined quantitatively by reaction with 0.2 % (w/v) ninhydrin in acetone at 100° for 10 min. Remaining cells of each type mixed, suspended in conjugation medium (glucose + MgSO₄ + phosphate), shaken for 5 hr. at 30°, then assayed microscopically for % conjugants as described in Methods.

Treatment	%	Ninhydrin reaction	
		Strain 5	Strain 21
None	64	++	+++
1 hr. starvation	68	+	+
2 hr. starvation	61	+	+
4 hr. starvation	50	±	—
7 hr. starvation	8	—	—

Another experiment which confirmed the above results made use of the amino acid analogues *p*-fluorophenyl-alanine and ethionine which are specific antagonists of phenylalanine and methionine, respectively. Table 9 shows the effect of these substances.

Table 9. *Effect of amino acid antagonists on conjugation in Hansenula wingei*

Cells of two mating types mixed and suspended in conjugation medium (glucose + MgSO₄ + phosphate) containing various additions as indicated, shaken 5 hr. at 30°, then assayed microscopically for % conjugants as described in Methods.

Treatment	% Conjugation
None	66
Fluorophenylalanine, 0.02 M	0
Fluorophenylalanine, 0.02 M + phenylalanine, 0.02 M	75
Phenylalanine, 0.02 M	68
Ethionine, 0.02 M	24
Ethionine, 0.02 M + methionine, 0.02 M	67
Methionine, 0.02 M	69

From a consideration of these results, the following hypothesis seems tenable. When the two mating types are brought together, there is a reciprocal induction in each type by its opposite mating type of an enzyme which softens and eventually digests its own cell wall. Thus the two cells form a conjugation tube between them, and eventually their cytoplasm mix and fusion is completed. The enzyme which is induced may be similar to, or identical with, the normal budding enzyme responsible

for the small hole in the yeast wall through which the bud develops (Nickerson & Falcone, 1956). The only difference is that in the present case the enzyme does not act randomly on the wall, but always at the point where the two cells touch. Support for this hypothesis is shown in the following experiment, where one cell type stimulated budding in the opposite type under conditions in which fusion could not take place. U.v.-irradiated cells of one type were mixed with non-irradiated cells of the opposite type and incubated for 5 hr. This experiment was performed with cells irradiated for 60 sec., since this irradiation time completely inhibited conjugation. After incubation the cells were de-agglutinated as usual, and the number of budding cells counted and compared with the number of budding cells in controls incubated unmixed. In the results given in Table 10, the % of buds was calculated from only those cells which showed young buds indicative of new bud formation. It is impossible to know which buds are on strain 5 cells and which are on strain 21 cells, but since little bud formation occurs when normal or irradiated cells are incubated separately, or when irradiated cells of both types are incubated together, it seems clear that when irradiated cells of one type are incubated with unirradiated cells of the opposite type, the unirradiated cells are stimulated to bud. This might be due to induction of the conjugation (or budding) enzyme by contact with cells of the opposite mating type.

Table 10. *Number of buds on mated ultraviolet-treated cells of Hansenula wingei*

Cells were irradiated 1 min. (sufficient to diminish conjugation to less than 1 %), then mixed with irradiated or non-irradiated cells of opposite type as indicated, then suspended in conjugation medium (glucose + MgSO_4 + phosphate) and shaken 5 hr. at 30°. Cells then processed in the same way as in standard conjugation assay, and the % of budding cells determined microscopically. No detectable conjugation was seen.

	Buds (%)
Strain 5, u.v. + Strain 21, u.v.	8
Strain 5, u.v. + Strain 21, normal	29
Strain 5, normal + Strain 21, u.v.	24

DISCUSSION

These studies have revealed a number of new points about the process of conjugation. In the following discussion, conjugation is defined as the process of cell fusion and implies nothing about nuclear interactions or other related events.

In *Hansenula wingei* conjugation occurs only when the two cells of the mating pair are in contact with each other. Conjugation will occur in the absence of growth, in a medium containing only an energy source, magnesium sulphate and potassium phosphate. The addition of nitrogenous compounds is of no benefit to the fusion process. Inhibitors which prevent energy metabolism prevent the fusion process.

Both cell types must function in the fusion process, and fusion will be inhibited when either of the two cells of the mating pair is treated with u.v. radiation or with cycloheximide. The process therefore differs from mating in *Escherichia coli* (Wollman, Jacob & Hayes, 1956) which appears to require only one of the mating pairs, the DNA donor, to be active.

Synthesis of new protein is required for fusion to occur, and this synthesis of protein can be inhibited by amino acid analogues. The precursors for the synthesis of

this new protein probably come from the free amino acid pool. It seems reasonable to assume that the new protein synthesized is a wall-softening enzyme, the synthesis of which is induced in each cell by contact with its mate. Evidence for this enzyme has been advanced by showing that when a cell is in contact with an u.v.-treated cell of the opposite type, the untreated cell is induced to bud, whereas new buds do not normally occur in the conjugation medium. The hypothesis is that the wall-softening enzyme presumed to be involved in cell fusion is similar to, or identical with, that involved in normal budding, and that during conjugation it is synthesized in large amounts, its action being localized at the point of contact of the mating cells. Thus conjugation can be viewed as an extension of the normal budding process. The fact that the first bud of the conjugant always occurs at the point of the conjugation tube midway between the two cells, where the wall is presumably the softest, is in keeping with this hypothesis (see Fig. 2).

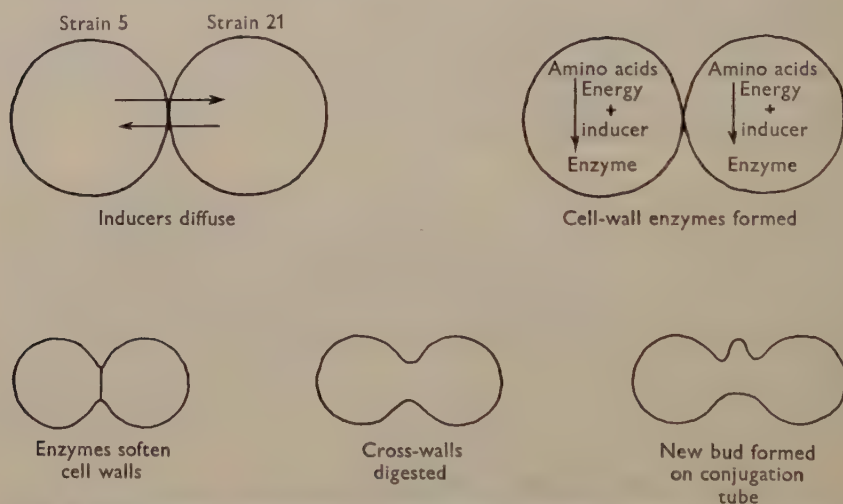


Fig. 2. Hypothetical explanation of physiological processes involved in cell fusion.

Hansenula wingei is an ideal organism for studying the physiology of conjugation, since the strong attraction between cells of opposite mating types makes it possible to aerate agglutinated cells extensively and to handle them easily in physiological experiments.

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Illegitimate Mating in *Paramecium bursaria* and the Basis for Cell Union

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SUMMARY

Cells from stock Wu-67 of *Paramecium bursaria*, syngen 1, can be induced to conjugate *inter se* (self) if they have made brief but transitory contact with cells of a complementary mating type; in mixtures consisting of marked cells of Wu-67 and cells of a complementary mating type both interclonal and intraclonal (Wu-67 \times Wu-67) conjugations were recognized. Unmixed cultures of Wu-67 never self nor could selfing be induced in other ways. In contrast to normal conjugation the intraclonal matings are illegitimate because they occur between cells expressing a common mating-type specificity. These and other facts are considered in light of Weiss's hypothesis about cell unions; it is postulated that a primary specific surface reaction dependent upon mating-type complementarity serves to elicit a secondary non-specific ('holdfast') reaction leading to the completion of conjugation. In cells from Wu-67 the sites of secondary reaction are held to be precociously or more readily activated than in normal cells and, when two such activated cells are apposed, illegitimate mating may occur. Under certain circumstances then, cellular adhesion and conjugation may be independent of primary mating-type complementarity and dependent instead upon the availability of secondary non-specific attachment sites.

INTRODUCTION

In *Paramecium* conjugation takes place when sexually mature cells of complementary mating types are brought together under appropriate environmental conditions. Although cells which belong to a common mating type do not normally conjugate among themselves, and reproductive isolation is the primary basis for the definition of physiological species (syngens or varieties), exceptions to these rules have been noted by various workers. Such findings are of intrinsic interest but they also seem pertinent to more general problems such as the basis for adhesions among somatic cells and the nature of sexuality—how do artificially dissociated somatic cells become properly reoriented and how are cellular changes leading to meiosis and fertilization induced? The observations and experiments reported in the present paper are concerned with illegitimate (intraclonal) conjugations in a mutant clone of *Paramecium bursaria*. The data are consistent with an hypothesis put forward by Weiss (1960), for they suggest that the cellular pairing, which leads to conjugation, is normally a consequence of a primary specificity and a secondary non-specific interaction of cell surfaces.

Previous investigations reported by Sonneborn (1942), Metz (1954) and Hiwatashi (1955) led to the conclusion that the mating reaction in *Paramecium* involves two

distinct and separable mechanisms. In analyses of the primary specific mating reaction mechanism, Metz and co-workers and Hiwatashi showed that cells belonging to a single clone can mate amongst themselves after some of the individuals have made transient contacts with cells of a complementary mating type. The most recent and provocative work along these lines is that of Hiwatashi (1958) and Miyake (1956, 1958, 1960) who induced conjugations among normally incompatible cells of *Paramecium caudatum*, *P. aurelia* and *P. multimicronucleatum* by the use of chemical agents. In all of these studies, the question arises as to whether or not contact with a complementary cell or chemical treatment causes a specific change in mating type. If a change in mating type does occur, induced intraclonal matings may occur between animals of complementary mating types and should be termed legitimate. One contribution of the present analysis is the resolution of this question for a particular case. A brief report of these results has appeared in abstract form (Siegel & Larison, 1960).

METHODS

Standard clones of *Paramecium bursaria*, representing each of the four complementary mating types (designated A, B, C, D) known for syngen 1 of this species and described in detail elsewhere (Siegel, 1958; Siegel & Larison, 1960) were used in the present work. Stocks Wu-67, JP-55 and NC-64 were kindly furnished by Professor T. T. Chen. In *P. bursaria*, each animal cell normally carries several hundred chlorella cells in hereditary endosymbiosis giving the paramecium a green appearance. When cultures of *P. bursaria* are allowed to reproduce rapidly in darkness the fission rate of the paramecia will exceed that of the symbiotic algae, and the number of algae/animal will rapidly diminish until sublines lacking algae are produced (Siegel, 1960). For convenience we shall designate such cells as 'white'. Separate cultures consisting of parasitized (green) and of asymbiotic or chlorella-less (white) paramecia, readily distinguishable from one another, were prepared for each stock and used in the detection of legitimate interclonal and illegitimate intraclonal conjugations. When a culture of green cells of one mating type is mixed with a culture of white cells of another mating type, green \times white pairings are interclonal whereas green \times green and white \times white pairings are intraclonal. The sexuality of the paramecia is known to be independent of the presence of endosymbionts (Ehret, 1953). The origins and properties of the stocks and their derived sublines are listed in Table 1.

It should be emphasized that these stocks will conjugate only when mixed with sexually reactive cells of a complementary type. The capacity of stock Wu-67 to undergo illegitimate (Wu-67 \times Wu-67) as well as legitimate (interstock) conjugation following its mixture with a clone of a complementary mating type is described in a later section. The occurrence of illegitimate pairing was found to be independent of the symbiotic association.

Phase-contrast microscopical examination of living paramecia from stock Wu-67 showed them to be cytologically normal, containing a single micronucleus and a single macronucleus, two contractile vacuoles, gullet, etc. (see Wichterman, 1953).

Methods for cultivating and handling *Paramecium bursaria* as already described (Jennings, 1939; Ehret, 1953; Siegel, 1960) were used; they follow closely the standard procedures for *P. aurelia* (Sonneborn, 1950). Experimental cells were

obtained from mass cultures previously grown at 25° and subjected to diurnal periods of artificial white light (100 ft. candles). Under these conditions the cells reached a peak of sexual reactivity at noon (Ehret, 1953) and matings were initiated at that time. The mixtures were kept at 25° and observed for conjugations at 4 p.m. of the same day.

Mating types of unknown cultures were determined by testing separate samples of each with cells of the four standard mating types. The unknown was classified as a particular type if it failed to mate with the standard of that type, but was able to mate with cells of the remaining three types.

Table 1. *The properties, origins and designations of the stocks of Paramecium bursaria*

Stock	Natural source	Mating type	Endosymbiotic chlorella	Designation
8	Malibu Lake, California	A	Present	8 A-g
			Absent	8 A-w
Wu-67	Shanghai, China	A	Present	Wu-67 A-g
			Absent	Wu-67 A-w
25	Malibu Lake, California	B	Present	25 B-g
			Absent	25 B-w
32	Malibu Lake, California	C	Present	32 C-g
			Absent	32 C-w
34	Fish Canyon, California	C	Present	34 C-g
			Absent	34 C-w
JP-55	Sapporo, Japan	C	Present	JP-55 C-g
			Absent	JP-55 C-w
3	Malibu Lake, California	D	Present	3 D-g
			Absent	3 D-w
NC-64	North Carolina	D	Present	NC-64 D-g
			Absent	NC-64 D-w

RESULTS

The initial observation on conjugations involving cells from stock Wu-67 demonstrated their unique ability to mate among themselves. Table 2 shows the results of mixing representative stocks of syngen 1 in all possible combinations of twos; in each combination, one of the cultures consisted of green cells while the other consisted of white cells; thus the origin of each paired cell could be ascertained. As expected, in mixtures of cultures belonging to a common mating type no pairing occurred, while in mixtures of cells of complementary mating types numerous pairs formed, each pair consisting of a cell from each of the two complementary stocks. In striking contrast mixtures of cells from stock Wu-67 with cells of a complementary mating type always yielded exceptional pairs, both mates originating from stock Wu-67, in addition to interstock pairs. Such illegitimate pairing never occurred in unmixed control cultures of stock Wu-67.

Detailed observations were made on those mating reactions which led to the formation of Wu-67 × Wu-67 pairs. The cells of stock Wu-67 were readily distinguished from those of the complementary mating type by the presence of chlorellae. Upon mixing cultures of complementary mating types, the usual swimming movements serve to bring two individuals into accidental contact; the cells immediately

'stick' if they belong to complementary types. These initial unions involve any part of the ciliated surface and so are irregular; they were easily disrupted by picking up and then expelling the cells with the aid of a micropipette. In agreement with the observations of others, a third and fourth cell were seen to adhere to the two initial cells and this continued until, a few minutes after mixture, large masses containing 100 or more individuals were formed. When the clumps were artificially broken up soon after they had formed, many loosely joined pairs were observed. Since neither green \times green nor white \times white unions were found, it must be con-

Table 2. The occurrence of interclonal and intraclonal conjugation among stocks representing the four mating types of *Paramecium bursaria*, syngen I. Cells were 'marked' by the presence (green) or absence (white) of symbiotic algae

		Cells contain chlorellae—'green'							
		A		B	C			D	
		Wu-67 A-g	8 A-g	25 B-g	32 C-g	34 C-g	JP-55 C-g	3 D-g	NC-64 D-g
Cells lack chlorellae—'white'	Mating type	Stock							
	A	Wu-67 A-w	—	—	GW	GW	GW	GW	GW
		8 A-w	—	—	WW	WW	WW	WW	WW
	B	25 B-w	GG	.	—
			GW	GW	GW	GW	GW	GW	GW
	C	32 C-w	GG
			GW	GW	—	—	—	GW	GW
		34 C-w	GG
			GW	GW	—	—	—	GW	GW
	D	JP-55 C-w	GG
			GW	GW	—	—	—	GW	GW
		3 D-w	GG
			GW	GW	GW	GW	GW	—	—
		NC-64 D-w	GG
			GW	GW	GW	GW	GW	—	—

'WW' indicates the presence of pairs consisting of two white cells, hence intraclonal conjugation.

'GG' indicates the presence of pairs consisting of two green cells, hence intraclonal conjugation.

'GW' indicates the presence of pairs consisting of one white cell and one green cell, hence interclonal conjugation.

'—' indicates an absence of pairs, hence stocks belong to the same mating type.

cluded that only cells of complementary types adhered within clumps. During the first 2 hr., the events of the mating reaction followed the normal pattern detailed by others (see Jennings, 1939; Wichterman, 1953). About 2 hr. after the initiation of the mating, the cells began to lose their ciliary stickiness and the large clumps disintegrated into pairs, each cell tightly joined to its mate along the opposing oral surfaces. Now for the first time illegitimate (green \times green) as well as legitimate (green \times white) pairs were observed. Unlike those formed earlier, these pairs were joined by 'holdfast unions' (Metz, 1947) and most of them could not be forced apart. The point to be stressed is this: legitimate interclonal conjugation proceeds from loose initial loose ciliary agglutinations to the formation of tightly joined and properly oriented pairs. However, intraclonal mating (Wu-67 \times Wu-67) apparently occurs directly, omitting the stage of initial ciliary agglutinations; as will be shown

later, it is dependent only on prior *interclonal* cell unions. Although cells of a given mating type are frequently packed next to one another during clumping, loose and randomly oriented *intraclonal* unions were not observed. Since the interaction of cells expressing complementary mating types results in loose ciliary unions and since no such unions were observed between two cells of stock Wu-67, these *intraclonal* conjugations might be interpreted as illegitimate. The two experiments next to be described provide further evidence for this conclusion.

In the first experiment, a sexually reactive culture of Wu-67-g was mixed with a *chlorella-less* culture of a complementary type. The Wu-67 \times Wu-67 (green \times green) pairs were isolated and immediately forced apart by repeatedly expelling the pair from a micropipette (Sonneborn, 1950). The viable members of such split pairs were re-isolated, allowed to multiply to form large cultures and subsequently tested for mating type. (As mentioned before, many pairs are firmly united and cannot be forced apart successfully; thus a certain amount of selection enters into the choice of cells to be tested.) All such cultures were found to consist exclusively of cells of mating type A. Thus there was no evidence for an hereditary change of mating type associated with the induction of selfing.

In the second experiment split pairs were obtained in the manner described above, but immediately after the pairs had been disjoined mating-type tests on each member of the pair were begun. Each uncoupled cell was placed first in a culture of sexually reactive *chlorella-less* testers of mating type B. As soon as an initial mating reaction (ciliary agglutination) was observed between the green (Wu-67) member of the split pair and a white cell (or cells) of the complementary mating type, the Wu-67 cell was removed and placed in another drop, this containing white cells of type C. When the Wu-67 cell reacted with cells of type C, it was again removed and placed with cells of type D, and finally with cells of type A. Such a series of four tests was completed within 20 min. after the original Wu-67 \times Wu-67 pair was split. The results appear in Table 3. Some cells could not be fully tested. Several died, apparently due to injury incurred during the process of separation. In other cases a cell from a split pair failed to react with two of the standard mating types, but this was not unexpected because the original pairs were obtained from clumps which were disintegrating due to the gradual loss of initial ciliary mating reactivity. These incomplete tests (split pairs numbered 6-10) provided no data contrary to those furnished by the cells which gave complete sets of tests. None of the cells showed a mating reaction with cells of type A; therefore none can be classified as belonging to a mating type other than A.

The frequency of contact between cells of stock Wu-67 and individuals of a complementary mating type was varied by altering the cell population density (but not their total numbers) in reaction mixtures. The data in Table 4 show that both legitimate and illegitimate conjugations were favoured by higher cellular concentrations. However, in denser populations about 12% of all conjugations were illegitimate, while less than 2% of the pairs were illegitimate in more sparse populations. In another experiment, the ratio of Wu-67 cells to those of a complementary type (32C-w) was varied while the total population density was kept constant. It was immediately apparent that in mixtures wherein cells of one mating type are present in excess numbers, the masses of clumped cells which formed soon after mixture contained an excess of individuals of that type. When there was an

Table 3. *The mating types of cells from uncoupled Wu-67 × Wu-67 pairs. Each ex-pair member (I and II) was tested against standard types A, B, C, D*

(Key: '+' = mating reaction; '-' = no reaction; '0' = cell died or was lost.)

Split pair	Cell	Reaction with testers of the indicated mating type			
		A	B	C	D
1	I	—	+	+	+
	II	—	+	+	+
2	I	—	+	+	+
	II	—	+	+	+
3	I	—	+	+	+
	II	—	+	+	+
4	I	—	+	+	+
	II	—	+	+	+
5	I	—	+	+	+
	II	—	+	+	+
6	I	0	+	+	+
	II	0	+	+	0
7	I	—	+	+	+
	II	—	+	+	—
8	I	0	+	+	0
	II	0	+	0	0
9	I	—	+	—	—
	II	—	+	—	+
10	I	—	—	+	+
	II	—	+	+	—

excess of 32C-w over Wu-67 cells, the frequency of contacts between *given* individuals of Wu-67 and cells of the complementary mating type was greater than that occurring in mixtures set up with an excess of Wu-67 cells. The results summarized in Table 5 are consistent with the idea that the effect of more frequent contacts with complementary cells, brought about by the mating reaction, was to increase the incidence of illegitimate conjugations.

Table 4. *The effect of population density on the frequency of illegitimate conjugation (Wu-67 × Wu-67 pairs)*

No. cells per ml. in mixtures Wu-67 A-g × 25 B-w	Expt.	Number of pairs	
		Illegitimate (green × green)	Legitimate (green × white)
340	1	5	76
	2	5	45
	3	14	56
	4	8	54
	Total	32	231
170	1	1	25
	2	0	30
	3	1	38
	4	0	36
	Total	2	139

All attempts to induce illegitimate Wu-67 \times Wu-67 conjugations by breis or cell-free fluids from cultures of complementary types were unsuccessful. Nor could we find evidence for the existence of an 'inducing substance' released by sexually-reacting cells. Sexual reactivity is a requisite for induced selfing; unreactive Wu-67 animals could not be induced to self, and unreactive cells of a complementary type did not induce illegitimate mating. Finally, when reactive cells of Wu-67 were mixed with reactive cells of a non-complementary mating type (mating type E, syngen 2), neither interstock nor Wu-67 conjugations occurred.

Genetic analyses of stock Wu-67 were virtually ruled out by the unfortunate fact that crosses involving this stock yield extremely high proportions of non-viable progeny (see Table 6).

Table 5. *The effect of population composition on the frequency of illegitimate conjugation (Wu-67 \times Wu-67 pairs)*

Approx. no. of cells mixed		Number of pairs		Illegitimate pairs (%)
Wu-67 A-g	32 C-w	Illegitimate (green \times green)	Legitimate (green \times white)	
1200	200	0	165	0
700	700	6	147	4
200	1200	5	58	8

Table 6. *The viability of exconjugant clones from legitimate and illegitimate matings involving stock Wu-67*

Mating	No. clones studied	No. clones viable
Wu-67 A-g \times 25 B-w	282	3
Wu-67 A-g \times 32 C-w	84	0
Wu-67 A-g \times 34 C-w	110	1
Wu-67 A-g \times 3 D-w	88	0
Wu-67 \times Wu-67	180	2

DISCUSSION

Stock Wu-67 is the only stock of *Paramecium bursaria* known to form pairs *inter se* following contacts with cells of complementary mating types. These intraclonal pairings can be termed illegitimate, for they evidently occur between cells expressing a common mating type. In sharp contrast, the intraclonal conjugations previously reported for *P. bursaria* (Jennings, 1941), *P. aurelia* (Kimball, 1939; Sonneborn, 1947), *P. multimicronucleatum* (Sonneborn, 1957) and *Tetrahymena pyriformis* (Nanney & Caughey, 1955) always resulted from the differentiation of individuals of complementary mating types within the selfing clone. Such conjugation does not differ in principle from the more familiar interclonal matings and may be termed legitimate. (Certain selfing clones of *Tetrahymena* described by Elliott & Nanney, 1952, do not conform to this pattern.)

Weiss's (1960) model for cell specificity and selectivity appears to account for both legitimate and illegitimate conjugations in *Paramecium*. This model is based on a dualistic principle of action involving: (1) a specific primary reaction which serves to expose sites of reactivity by alteration of the cell surface; (2) a non-specific

secondary reaction at the level of the exposed sites. The model assumes that the cell surface contains a network of protein molecules which acts as a 'barrier' to cell union, transport and transmission. However, specific end groups on the protein molecules react with complementary groups on an extraneous carrier (particle or cell) and thus are reoriented from a tangential position to one radial to the cell surface. This reorientation causes 'breaches' in the barrier allowing the passage of materials or currents. Metz (1954) showed that cells of complementary mating types possessed specific and complementary sex substances on their surfaces whose union or interaction led to the initial adhesion of potential conjugants and provided a basis for the observed specificity of the primary mating reaction. Weiss's model suggests the next events of conjugation. The interaction of the specific sex substances results in their reorientation thereby exposing secondary non-specific holdfast sites; then, in accord with many observations, cells united at holdfast sites normally proceed through conjugation.

Weiss's model provides an explanation for the well-documented fact that paramecia which unite in conjugation belong to complementary types, for the initial intimate contact brought about by the specific interactions of surface molecules serves to insure heterotypic holdfast unions. By assuming that the non-specific holdfast sites of cells from stock Wu-67 are more readily (or earlier) exposed by ciliary contact with a legitimate prospective mate, illegitimate unions will be expected to occur when two Wu-67 cells are fortuitously pressed together during the mass clumping phase of mating.

The hypothesis seems to account in a satisfactory way for the facts concerning stock Wu-67. Firstly, a mating reaction involving cells of Wu-67 and a complementary type is a necessary condition for illegitimate conjugation. Secondly, cells of Wu-67 which have begun to conjugate illegitimately are unchanged with respect to mating type specificity (all are type A). Thirdly, Wu-67 \times Wu-67 pairs first appear when clumps disintegrate, and the mates are always united by one or more of the three holdfast sites known for *Paramecium*. The fact that Wu-67 \times Wu-67 pairs are more frequent in mixtures in which there is an excess of cells of a complementary type may mean that Wu-67 cells must be in repeated or prolonged contact with cells of a complementary type in order to precociously uncover the holdfast site.

The immediate value of the hypothesis is that it appears to account for a number of observations concerning irregular mating reactions in ciliates. For example, the hypothesis predicts that a block might occur between the primary specific reaction and the secondary non-specific reaction. As Sonneborn (1942) and Metz (1954) stressed, the behaviour of a mutant stock of *Paramecium aurelia* indicates the existence of discrete steps in the reactions leading to conjugation. A block CM (= 'can't mate') evidently occurs after the primary interaction of mating-type substances. Thus cell agglutination proceeds normally but the CM cells are unable to complete conjugation because the secondary non-specific sites are effectively unavailable.

Other observations reveal the fact that the initial cellular agglutinations which are brought about by mating-type specificity differences are not inevitably followed by the formation of tightly joined pairs and so are consistent with the notion of separable primary and secondary interactions in conjugation. For example, both nutritive condition and life-cycle stage are factors known to determine whether or

not the loose cellular aggregates characteristic of the early phase of mating will yield tightly joined pairs which complete conjugation.

Hiwatashi (1958) and Miyake (1958, 1960) showed that chemical agents can induce pair formation and conjugation in *Paramecium* irrespective of mating-type specificity. Particularly striking was the demonstration that chemicals induced matings between individuals of separate syngens and species, as well as intraclonal selfing. Following chemical treatment pairs were formed directly, the mates attached by their holdfast sites; since the initial stages of normal mating reactions (loose ciliary unions and the formation of clumps of reacting cells) are omitted, it does not seem likely that such conjugation is a consequence of specific mating-type changes. Chemical treatment may remove the barrier formed by molecules concerned with primary specific mating-type complementarity and so permit the secondary non-specific mating-type holdfast unions to occur directly.

Although the available data seem to fit Weiss's hypothesis, other kinds of explanations are not excluded. Dr T. M. Sonneborn in a personal communication, suggested that the primary specific mating reaction serves to trigger another *internal* cellular reaction which leads to the non-specific receptivity of the cortical surface of the cell; firm cell attachments and conjugation occur when the activated cortical areas of cells are brought together. On this view the secondary reaction is not a direct mechanical consequence of the primary reactions of complementary molecules as Weiss's model would hold. Future research should be directed towards an understanding of the relationship between the primary and secondary mating reactions.

Finally, it must be pointed out that the mechanism proposed here for illegitimate pairing in *Paramecium bursaria*, and for the chemical induction of selfing described by Hiwatashi and by Miyake for other species, may not be applicable to the induction of pseudo-selfing reported by Sonneborn and by Metz. The latter workers were able to show that cells which have been temporarily united with individuals of a complementary mating type behaved as if they had acquired that mating-type specificity in the course of contact. Thus a transitory shift of mating type may occur in these cases. But in *P. bursaria* both mates need to be 'activated' by contact with cells expressing a complementary type in order that illegitimate conjugation may occur; this is certainly not the case for the pseudo-selfers of Sonneborn and Metz. Although the phenomena may appear superficially similar they probably call for quite different explanations.

Induced selfing of the kind reported for stock Wu-67 is apparently extremely rare in *Paramecium bursaria*. No evidence for this phenomenon was observed among at least 100 clones studied by Jennings (see Sonneborn, 1947) and others (Ehret, 1953; Siegel, 1960; Wichertman, 1953). Therefore homotypic matings are presumably unimportant in the evolution and genetic analyses of this organism.

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Resistance and Cross-resistance of *Escherichia coli* Mutants to Antitumour Agent Mitomycin C

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SUMMARY

The cross-resistance patterns are described for 96 mutants selected in one step from *Escherichia coli* strain S for resistance to Mitomycin C. The test agents used were ultraviolet radiation, seven radiomimetic and two non-radiomimetic compounds. Seven different types of mutants could be selected in one step from the parent. Five of these were radioresistant; two were chemoresistant. Of the radioresistant types two were identical with types previously isolated using other radiomimetic agents for selection; three of the types were new. One of the new types was reactivated by plating medium following ultraviolet irradiation differently from all other radioresistant mutants of *E. coli* S. One of the chemoresistant types was resistant only to Mitomycin C; the other displayed a low degree of cross-resistance to nitronitrosoguanidines and to penicillin. Also described are the cross-resistance patterns of mutants selected in five consecutive steps for increasing resistance to Mitomycin C. Beginning with a first step radioresistant mutant it was possible to select four additional steps, up to 325-fold, in resistance to Mitomycin C. One of these steps appeared to be a shift from one radioresistant type to another.

INTRODUCTION

This paper describes the cross-resistance patterns of mutants of *Escherichia coli* strain S, selected for resistance to Mitomycin C, an antibiotic with antitumour activity (Usubuchi *et al.* 1957). Mitomycin C has radiomimetic properties: in bacteria it is a mutagen (Szybalski, 1958) and like radiation it selectively inhibits the synthesis of deoxyribonucleic acid (Shiba, Terawaki, Taguchi & Kawamata, 1959). Mutants of *E. coli* S selected for resistance to other radiomimetic agents were cross-resistant to Mitomycin C (Mandell, Woody & Greenberg, 1961; Woody, Mandell & Greenberg, 1961; Greenberg, Mandell & Woody, 1961). Furthermore, Oboshi (1959) found that tumours selected for resistance to nitrogen mustard N-oxide (nitromin) were also resistant to Mitomycin C.

In earlier work, 13 first-step mutants of *Escherichia coli* S were selected for resistance to 1-methyl-3-nitro-1-nitrosoguanidine (Mandell *et al.* 1961), to nitrogen mustard and to nitromin (Woody *et al.* 1961), to azaserine, to 1-chloropropyl- and 1-chloroethyl-3-nitro-1-nitrosoguanidine (Greenberg *et al.* 1961). Each mutant was characterized by its cross-resistance pattern, i.e. its degree of resistance to ultraviolet radiation, seven radiomimetic and two non-radiomimetic agents (penicillin

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and 6-diazo-5-oxo-L-norleucine). The cross-resistance patterns of the 13 mutants are shown in Table 1. Three of the mutants (S/Ni 1a, S/As 1a and S/Cp 1b) were significantly resistant only to the selecting agent or to closely related chemicals. Such mutants have been termed 'chemoresistant' to indicate that resistance is to a specific chemical structure and not to the class of radiomimetic agents. The other ten mutants were resistant to all radiomimetic agents and to ultraviolet radiation but not to the non-radiomimetic agents. These have been called 'radioresistant' mutants (with the limitation that the effect of ionizing radiations on these mutants has not yet been tested). Four different cross-resistance patterns were observed among the 10 radioresistant mutants; these have been designated R₁, R₂, R₃ and R₄. R₁ and R₂ patterns were observed once each; R₃ and R₄ patterns occurred in mutants selected for resistance to three and five, respectively, different chemical agents. All

Table 1. *Cross-resistance among first step mutants resistant to radiomimetic compounds*

Bacterial strain	Radio-resistant designation	Test compound									u.v.
		MC*	NG	CP	CE	NM	NI	AS	DON	PN	
		Minimum inhibitory concentration for <i>Escherichia coli</i> S†									
		0.038	0.038	0.018	0.021	13	18	0.004	0.04	2.2	
		±	±	±	±	±	±	±	±	±	
		0.005	0.006	0.002	0.003	2	3	0.001	0.02	0.2	
Resistance factor‡											
T 7.0 M											
S/Ni 1a§	—	1	1.2	1	1	1.2	60	1	1	1	1
S/As 1a	—	1	0.7	1	1	1	1	16	1	1	1
S/Cp 1b	—	1.2	12	9	9.5	0.18	0.22	1.1	1	1	1
S/Cp 1a	R ₁	2.7	2.0	2.3	1.6	2.9	60	4.0	1	1	15
S/Ni 1b	R ₂	5.8	19	6.5	6.2	6.2	60	6.8	1	0.34	15
S/Nm 1a	R ₃	9	28	9	9.5	10	60	13	1	1	15
S/Ni 1c											
S/As 1b											
S/Ng 1a	R ₄	22	45	26	24	20	60	16	1	1	15
S/Nm 1b											
S/As 1c											
S/Ce 1a											
S/Cp 1c											

* The following abbreviations will be used: Mitomycin C, MC; 1-methyl-3-nitro-1-nitrosoguanidine, NG; 1-chlorethyl-3-nitro-1-nitrosoguanidine, CE; 1-chloropropyl-3-nitro-nitrosoguanidine, CP; nitrogen mustard, NM; nitromin, NI; azaserine, AS; 6-diazo-5-oxo-L-norleucine, DON; penicillin, PN; ultraviolet light, u.v.

† $\mu\text{g./ml.}$ estimated from gradient plates (Szybalski & Bryson, 1952) except the u.v. value is expressed in $\text{ergs mm.}^{-2}/\text{hit}$ based on calibration of the u.v. source with T2 phage (Latarjet *et al.* 1953). 'Hit' is defined as $-\ln$ (fraction of survivors). The survivors were plated on tryptone agar pH 7.0 and M-9 minimal agar pH 6.8; the fold-increase, an average of several determinations, represents a comparison with the survival of the parent strain (plated on tryptone agar pH 7.0) set at unity.

‡ The figures in these columns represent the factor of resistance (fold-increase) compared with the parent strain set at unity.

§ Resistance to specific agents is conventionally represented by a bar and the abbreviation of the agent to which the strain was first found resistant. The first number following the bar denotes the selective isolation step to resistance; subsequent letters and numbers identify particular mutants and their families.

the radioresistant mutants exhibited an identical degree of resistance to ultraviolet light, unaffected by post-irradiation growth on defined or complex media. This is in contrast to *E. coli* B (Roberts & Aldous, 1949) and its radioresistant mutant, B/r (Alper & Gillies, 1960; Hill & Simson, 1961) and to *E. coli* S (Woody *et al.* 1961) whose degree of resistance to ultraviolet radiation can be altered by the post-irradiation growth medium. All the radioresistant mutants were equally resistant to nitroimin, but differed in their degree of resistance to the other six radiomimetic agents. The R_1 mutant was least resistant to these agents, the R_4 mutants were most resistant, while R_2 and R_3 mutants were intermediate in their resistance. If only ultraviolet radiation and nitroimin had been used to characterize these mutants, they would all have appeared identical. However, by using nine test compounds, four different cross-resistance patterns were observed, indicating four different types of radioresistant mutant.

The results of an analysis of 96 first-step and four higher-step mutants show that when Mitomycin C is used as the selecting agent: (1) radioresistant mutants of the classes R_3 and R_4 occurred frequently, while representatives of the classes R_1 and R_2 were not isolated; (2) three radioresistant mutants with hitherto undetected cross-resistance patterns were isolated, including one whose resistance to ultraviolet radiation could be altered by the nature of the post-irradiation growth medium; (3) two types of chemoresistant mutants were isolated—one resistant only to Mitomycin C, the other with a low degree of cross-resistance to nitrosoguanidines and to penicillin and under certain circumstances to ultraviolet radiation; (4) it was possible to select five consecutive steps of increasing resistance, up to 325-fold, to Mitomycin C; (5) the second step conferred increased resistance to Mitomycin C without otherwise effecting the cross-resistance pattern of the first-step radioresistant parent; (6) an R_4 -type mutant was derived in a single selective step from a type R_3 parent.

METHODS

Bacterial strains. *Escherichia coli* strain S, obtained from Dr A. D. Hershey, was the parent strain. The characteristics of the resistant mutants used as reference strains are given in the appropriate tables. Their derivation is described in earlier papers (Mandell *et al.* 1961; Woody *et al.* 1961).

Compounds. The chemicals used were: 1-methyl-3-nitro-1-nitrosoguanidine, purchased from the Aldrich Chemical Co., Milwaukee, Wisc., and recrystallized from ethanol; 1-chloropropyl- and 1-chlorethyl-3-nitro-1-nitrosoguanidine kindly furnished by Dr B. R. Baker, Stanford Research Institute, Menlo Park, California; azaserine, 6-diazo-5-oxo-L-norleucine, Mitomycin C and nitroimin, supplied by the Cancer Chemotherapy National Service Center, Bethesda, Md.; and nitrogen mustard, a gift from Merck Sharp and Dohme, Rahway, N.J. All compounds were prepared in sterile distilled water immediately before use.

Media. The media used contained per litre of distilled water:

Tryptone agar. Tryptone, 10 g.; glucose, 1 g.; sodium citrate, 2.0 g.; sodium chloride, 8 g. and agar, 12 g. (BBL, Baltimore Biological Lab., Inc.); adjusted to pH 7.0 with sodium hydroxide or 5.5 with hydrochloric acid.

M-9 agar. Dibasic sodium phosphate, 5.8 g.; monobasic potassium phosphate, 3.0 g.; ammonium chloride, 1.0 g.; sodium chloride, 0.5 g.; glucose, 2.0 g.; mag-

nesium sulphate ($7\text{H}_2\text{O}$), 250 mg.; calcium chloride, 14 mg.; 1% gelatin solution, 10 ml.; agar, 8.0 g. (Ionagar, Oxo Ltd., London).

Peptone broth. Peptone, 10 g.; beef extract, 3 g.; glucose, 1 g.; sodium chloride, 5 g.

Diluting fluid. Peptone, 2 g.; sodium chloride, 6 g.; magnesium sulphate ($7\text{H}_2\text{O}$), 0.5 g.

Phosphate-buffered saline was 1% sodium chloride in 0.2 M-phosphate buffer, pH 6.8. Tryptone glucose extract agar was a commercial (Difco) preparation, on which cultures were preserved after isolation and identification.

Isolation of resistant mutants. Resistant mutants were isolated from plates of tryptone agar (pH 5.5) containing graded doses over a 100-fold range of Mitomycin C and spread with 3.5×10^7 organisms of the appropriate parent, growing logarithmically in peptone broth.

Measurement of resistance to chemical agents. The methods used to measure the degree of resistance to chemical agents have been given in detail elsewhere (Mandell *et al.* 1961). All isolated clones were grown overnight in peptone broth at 37° , adjusted turbidimetrically to a population density of about 3.5×10^8 /ml., and streaked on gradient plates, according to the method of Szybalski & Bryson (1952). Gradient plates were made with tryptone agar (pH 5.5) except that M-9 agar was used in tests involving azaserine, 6-diazo-5-oxo-L-norleucine and penicillin. The minimum inhibitory concentration (MIC) was determined as follows:

$$\frac{\text{length of solid growth}}{\text{total length of streak}} \times \text{maximum concentration of test compound } (\mu\text{g./ml.}).$$

Sensitivity to ultraviolet radiation. The ultraviolet radiation source was a single 15 W. General Electric germicidal lamp with a maximum output at 2537 \AA ., calibrated with bacteriophage T2 according to the method of Latarjet, Morenne & Berger (1953). Cultures grown overnight in peptone broth were washed twice with phosphate buffered saline, diluted to about 2×10^6 bacteria/ml. in cold buffered saline (pH 6.8) and exposed in 50 mm. Petri dishes containing 1 ml. of bacterial suspension. Exposures were made at a distance of 51.5 cm. from the ultraviolet radiation source. The dish was agitated gently by hand throughout exposure. Appropriate dilutions in cold diluting fluid were plated in duplicate on both tryptone agar (pH 7.0) and M-9 agar, incubated for 24 and 48 hr., respectively, at 37° and counted. All manipulations subsequent to ultraviolet irradiation were carried out in subdued light to minimize photoreactivation. Sensitivity to ultraviolet radiation was calculated as $\text{ergs/mm.}^{-2}/\text{hit}$, a hit being defined as $-\ln$ (fraction of survivors).

RESULTS

First-step mutants. The results of a survey of 100 potential first-step mutants resistant to Mitomycin C are presented in Table 2. The cross-resistance patterns of the various types of mutants are given in Table 3.

Only four of the 100 isolates were indistinguishable from the parent strain S. The remainder showed some degree of resistance to Mitomycin C. These could be divided into two main groups: those which were cross-resistant with *all* radio-mimetic compounds (radioresistant) and those which were not (chemoresistant). The latter group could be subdivided into two subgroups. One, the prototype of

Table 2. *Classification of survivors after treating Escherichia coli S with Mitomycin C*

Mytomycin C (μ g./ml.)	Surviving colonies (18 hr.)	No. tested	Sensi- tive	No. of type							Chemo- resistant	
				Radioresistant							I	II
				R ₁	R ₂	R ₃ *	R ₄ *	R ₅ *	R ₆ *	R ₇ *		
0.03	Confluent growth											
0.08	300-400	25	4	0	0	8	1	0	1	0	7	4
0.15	200	25	0	0	0	13	11	1	0	0	0	0
0.30	60	30	0	0	0	13	15	2	0	0	0	0
0.50	15	9	0	0	0	0	8	0	0	1	0	0
0.75	5 (8†)	8	0	0	0	0	8	0	0	0	0	0
1.0	1 (3†)	3	0	0	0	0	3	0	0	0	0	0
3.0	0	—	—	—	—	—	—	—	—	—	—	—
Totals		100	4	0	0	34	46	3	1	1	7	4

* Cross-resistant pattern of representative strains of each type S/Mc 1g, S/Mc 1b, S/Mc 1f, S/Mc 1i, S/Mc 1h, S/Mc 1e, and S/Mc 1d, respectively, are shown on Table 1.

† After 42 hr.

which is S/Mc 1e, was resistant (2.5-fold) only to Mitomycin C and is therefore clearly chemoresistant. The other subgroup of which S/Mc 1d is representative, was cross-resistant with nitrosoguanidines and penicillin but not with any of the other chemical agents. The response of S/Mc 1d and S to ultraviolet radiation (Fig. 1) was identical when both were plated on tryptone agar subsequent to irradiation. When, however, both were plated on M-9 agar, the slope of the survival curve of S increased six-fold, but that of S/Mc 1d increased 10- to 12-fold. This is the first instance in which the difference between radio- and chemoresistance is not clearcut. For the present, mutants of this group have been classified as chemoresistant, with the recognition that this is an arbitrary classification of an anomalous strain.

Table 3. *Cross-resistance relationships among first-step Mitomycin C resistant mutants of Escherichia coli strain S relative to the sensitive parent strain*

Bacterial strain	Radio-resistant designation	Test compound									u.v.	
		MC*	NG	CP	CE	NM	NI	AS	DON	PN		
		Minimum inhibitory concentration for <i>Escherichia coli</i> S†										
		0.038	0.038	0.018	0.021	13	18	0.004	0.04	2.2		
		±	±	±	±	±	±	±	±	±		
		0.005	0.006	0.002	0.003	2	3	0.001	0.02	0.2		
		Resistance factor‡										22
									Plated on			
									T 7.0	M-9		
S/Mc 1d§	—	1.4	1.6	1.4	1.2	1	1	1	1	1.3	1	10
S/Mc 1e	—	2.5	1	1	1	1	1	1	1	1	1	6
S/Mc 1i	R ₆	2.0	3.7	2.7	2.0	2.9	60	4.0	1	1	6	15
S/Mc 1a	R ₃	9	28	9	9.5	10	60	13	1	1	15	15
S/Mc 1e	R ₅	14	36	11	13	20	60	16	1	1	15	15
S/Mc 1f	—	11	—	—	—	—	—	—	—	—	—	—
S/Mc 1b	R ₄	22	45	26	24	20	60	16	1	1	15	15
S/Mc 1h	R ₇	31	56	36	31	20	60	16	1	1	14	14

*, †, ‡, §. See footnotes to Table 1.

Five distinct types of radioresistant mutants were isolated. 54% of the radioresistant mutants, represented by S/Mc 1b, had a cross-resistance pattern identical with that of all previously isolated mutants of type R_4 ; 40%, represented by S/Mc 1g, were indistinguishable from other members of type R_3 . The remaining five radioresistant mutants had cross-resistance patterns not previously observed. S/Mc 1e, S/Mc 1f and S/Mc 1h were identical to R_4 in their degree of resistance to ultraviolet radiation, nitrogen mustard, nitroimin and azaserine, and in their lack of resistance to DON and penicillin. However, S/Mc 1e and S/Mc 1f were somewhat less resistant than R_4 to Mitomycin C and the nitrosoguanidines, and S/Mc 1h was

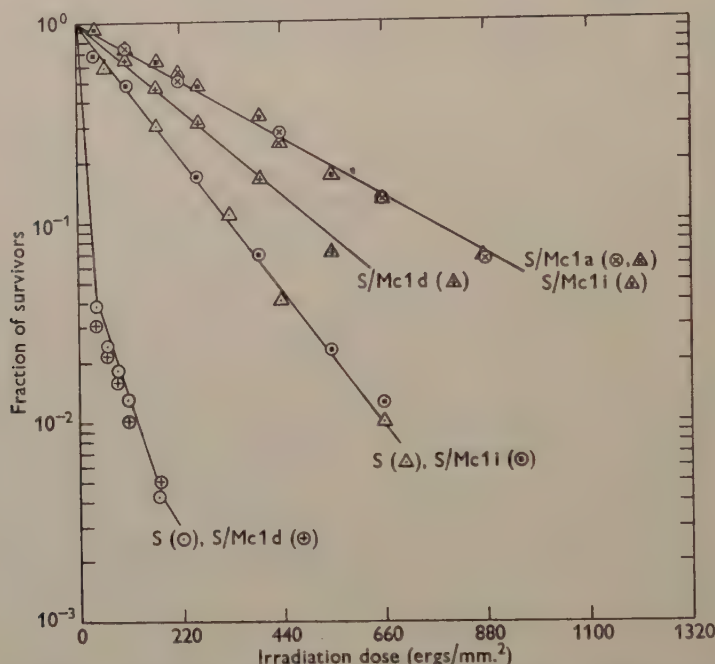


Fig. 1. Survival of *Escherichia coli* S, chemoresistant strain S/Mc 1d, and radioresistant strains S/Mc 1a and S/Mc 1i exposed to ultraviolet radiation. ○, Survivors plated on tryptone agar, pH 7.0. Δ, survivors plated on M-9 defined medium, pH 6.8.

somewhat more resistant than R_4 to these same compounds. S/Mc 1e and S/Mc 1f differed from each other only in a small, but seemingly consistent, manner on repeated trials, with regard to their resistance to Mitomycin C. They have been arbitrarily classed together as R_6 . S/Mc 1h has been designated R_7 .

The last, hitherto undescribed, radioresistant mutant, S/Mc 1i, had a cross-resistance pattern very similar to that of R_1 . However, unlike R_1 , which was 15-fold resistant to ultraviolet radiation irrespective of post-irradiation plating medium, S/Mc 1i was six-fold resistant when plated on complex (tryptone) medium and 15-fold resistant when plated on M-9 agar (Fig. 1). S/Mc 1i is, therefore, considered different from R_1 and has been designated R_6 .

The frequency distributions shown in Table 2 should be interpreted with caution. Nevertheless certain facts can be derived from the table. The frequency with which

a type of mutant was selected depended to some extent on the concentration of the drug. The mutants with the lowest degree of resistance to Mitomycin C were selected only from the plate with the lowest concentration of Mitomycin C at which there was not confluent growth. This plate was the only one from which parental, sensitive organisms were picked. As the concentration of Mitomycin C was increased, only the more resistant mutants were isolated. It would appear from the sampling of survivors over a 40-fold range of concentration of Mitomycin C that radioresistant mutants predominated and that most of these were R_3 or R_4 . However, half the mutant resistant population sampled from the plate containing Mitomycin C at 0.08 $\mu\text{g./ml.}$ were chemoresistant. It is curious that from this same plate only one R_4 -type mutant was isolated. Assuming no sampling prejudice, this would mean that there were 16 R_4 mutants on the plate, whereas at the next higher concentration of Mitomycin C there were 88 and the frequency decreased thereafter as the concentration of Mitomycin C increased. Actually the sampling of the 0.08 $\mu\text{g./ml.}$ plate was probably prejudiced, since there were several colony sizes and an almost equal number of each colony size was picked regardless of the true frequency of the various colony sizes on the plate. Only the mutants of the type S/Mc 1d and sensitives were found among the smallest colony type.

Table 4. *Cross-resistance relationships among mutants of Escherichia coli S representing five consecutive steps in resistance to Mitomycin C*

Bacterial strain	Test compound									Plated on		
	MC*	NG	CP	CE	NM	NI	AS	DON	PN			u.v.
	Minimum inhibitory concentrations for <i>Escherichia coli</i> S†											
	0.038	0.038	0.018	0.021	13	19	0.004	0.04	2.2			
	±	±	±	±	±	±	±	±	±	22		
	0.005	0.006	0.002	0.003	2	3	0.002	0.02	0.2			
Factors of resistance‡												
									T 7-0	M-9		
S/Mc 1a§	9	28	9	9.5	10	60	13	1	1	15	15	
S/Mc 2a	22	28	9	9.5	10	60	13	1	1	15	15	
S/Mc 3a	71	36	26	24	20	60	16	1	1.4	15	15	
S/Mc 4a	235	36	39	38	20	60	16	1	1.4	15	15	
S/Mc 5a	325	36	68	38	20	60	16	1	2.6	15	15	

*, †, ‡, §. See footnotes to Table 1.

Granting that the frequency of occurrence of any mutant type depends on the selective pressure of the Mitomycin C, nevertheless mutants of the types R_5 , R_6 and R_7 were rare compared to those of types R_3 and R_4 .

Sequential steps in resistance to Mitomycin C. Mutants representing five sequential steps in increasing resistance to Mitomycin C were selected as follows: S/Mc 1a was selected from S, S/Mc 2a from S/Mc 1a, S/Mc 3a from S/Mc 2a, etc., as seen in Table 4. The second step mutant, S/Mc 2a, was more resistant than its parent only to Mitomycin C. The third-step mutant, S/Mc 3a, increased in resistance to all radiomimetic agents. Its cross-resistance pattern was essentially that of an R_4 radioresistant mutant except that S/Mc 3a was more resistant to Mitomycin C than R_4 mutants. The fourth-step mutant increased in resistance not only to Mitomycin C

but also to 1-chloropropyl- and 1-chloroethyl-3-nitro-1-nitrosoguanidine. The fifth-step mutant increased in resistance to Mitomycin C, to 1-chloropropyl-3-nitro-1-nitrosoguanidine and to penicillin.

The survival curves of the second- to fifth-step mutants in Mitomycin C resistance, as well as those of R_1 , R_2 , R_4 , R_5 , and R_7 after exposure to ultraviolet radiation were indistinguishable from that of S/Mc 1a (Fig. 1) and were not altered by post-irradiation growth in complex or synthetic medium.

DISCUSSION

The original purpose of the work described here was to determine whether, by cross-resistance studies, relationships could be established among compounds which for various reasons (Mandell *et al.* 1961) were considered to be radiomimetic. It has been shown in this paper and previous papers of this series that there exists a class of compounds such that mutants of *Escherichia coli* S selected for resistance to one member of the class will be resistant to all members of the class and to ultraviolet radiation. Most of the mutants of *E. coli* S selected for resistance to 1-methyl-3-nitro-1-nitrosoguanidine (Mandell *et al.* 1961), nitrogen mustard and nitromin (Woody *et al.* 1961), azaserine and other nitrosoguanidines (Greenberg *et al.* 1961) and, as shown in this paper, Mitomycin C, were also resistant to ultraviolet radiation and to each of the other radiomimetic agents tested. This cross-resistance was not the result of a general increase in resistance to all antibacterial agents, for none of the radioresistant mutants of S was significantly resistant to 6-diazo-5-oxo-L-norleucine or penicillin. The observations regarding mutual cross-resistance among radiomimetic agents and radiation are extensions of earlier observations of Bryson (1948) who found that Witkin's (1947) strains of *E. coli* B, selected for resistance to radiation, were also resistant to nitrogen and sulphur mustard. Conversely, strains selected for resistance to nitrogen mustard were resistant to radiation (Bryson, 1948). Furthermore, the radiation-resistant mutant of *E. coli* B, strain B/r, was similarly found resistant to nitrofuracin, and strains selected for resistance to nitrofuracin were also resistant to radiation (Szybalski & Nelson, 1954).

However, not all mutants selected for resistance to radiomimetic compounds are radioresistant. It was shown earlier that one mutant selected for resistance to nitromin was resistant only to nitromin and not to other radiomimetic agents—not even the chemically similar nitrogen mustard—nor to radiation (Woody *et al.* 1961). It has been shown in this paper that there is at least one first-step mutant resistant to Mitomycin C (S/Mc 1c) which is resistant only to Mitomycin C. There was another mutant which could not so unequivocally be classified as chemoresistant. S/Mc 1d, which appeared as sensitive as the parent S to ultraviolet radiation when plated on complex medium, was reactivated to a much greater extent than S when the organisms were plated on M-9 agar. Furthermore, S/Mc 1d was resistant not only to Mitomycin C but was also resistant to the nitrosoguanidines and to penicillin.

There are other examples where resistance to Mitomycin C and one or more nitrosoguanidines increase or decrease together without any concomitant change in resistance to any of the other agents used in these studies. Mutants classified as R_5 and R_7 (Table 3) have cross-resistance patterns identical with R_4 except for their resistance to Mitomycin C and the nitrosoguanidines, R_5 being somewhat less and

R₇, somewhat more resistant to all these agents than R₄. S/Mc 4a and 5a (Table 4) were characterized by increases in resistance only to Mitomycin C and to one or more nitrosoguanidines; S/Ng 2a, a second-step mutant selected for resistance to 1-methyl-3-nitro-1-nitrosoguanidine displayed an increase in resistance to Mitomycin C (Mandell *et al.* 1961). These data suggest that Mitomycin C and the nitrosoguanidines may have in common a chemically reactive group or a mode of action, not necessarily radiomimetic. The elucidation of the chemical structure of Mitomycin C will tell whether the former possibility is true.

Seven different first-step radioresistant mutants have been isolated to date from the parent strain S. Two of these, R₃ and R₄, have been isolated repeatedly using a variety of different radiomimetic agents for selection. R₃- and R₄-types comprised the overwhelming majority of the radioresistant mutants isolated when Mitomycin C was the selecting agent (Table 2). The other five radioresistant types were rare, R₁, R₂, R₆ and R₇ having been selected once each from among mutants resistant to each of five different radiomimetic agents. Three mutants of the type R₅ were isolated when Mitomycin C was the selecting agent but not when any other radiomimetic agent was used. It should be pointed out, however, that the current survey of 100 potential resistant mutants is the most extensive we have undertaken. It is, therefore, impossible to decide whether R₁ was induced more frequently by 1-chloro-propyl-3-nitro-1-nitrosoguanidine, R₂ by nitroimin, and R₅, R₆ and R₇ by Mitomycin C, or whether, being rare, they were selected by these particular agents by chance. It is also difficult to decide whether the rare R-types are actually the result of single mutational events, or whether they represent double mutations. R₅ and R₇ might conceivably be R₃ and R₄, respectively, with a second, chemoresistant mutation to Mitomycin C resistance. R₁ and R₂, having such a low degree of resistance to all radiomimetic compounds, would have to be R₃ or R₄, modified by a second mutation to a lower degree of resistance to all radiomimetic compounds. R₆, differing as it does from all other R-types in its plating medium reactivation characteristics, is probably the product of a single mutation. Based on preliminary estimates of the frequency with which the presumed primary mutations to resistance occur ($c 3 \times 10^{-6}$ for R₃ and R₄ and the chemoresistant type of S/Mc 1c with Mitomycin C at 0.08–0.15 $\mu\text{g./ml.}$) it seems unlikely that double mutations would be selected from among the relatively small samples plated (3×10^7 on each of the Mitomycin C plates).

All the radioresistant mutants of *Escherichia coli* S differ in several ways from the radioresistant mutant of *E. coli* B, B/r (Witkin, 1947). The degree of resistance to ultraviolet radiation of all radioresistant types of S, except R₆, was unmodified by the type of medium on which they were plated following irradiation. *E. coli* B/r, on the other hand, appears to be more resistant than strain B to ultraviolet radiation only when both strains are plated on complex medium (Alper & Gillies, 1960). When both B and B/r were plated on defined medium following irradiation, they had indistinguishable survival curves. This is because B is more sensitive to irradiation when plated on complex medium than when plated on defined medium (Roberts & Aldous, 1949), while the opposite is true of B/r. *E. coli* S behaves with respect to plating medium reactivation like *E. coli* B; the radioresistant type R₆ behaves, qualitatively, like *E. coli* B and S, the opposite to B/r, and differently from all the other R-types of S.

B/r also differs from the R-types of *Escherichia coli* S with respect to collateral

resistance to penicillin and the sulphonamides. According to Witkin (1947) and Adler & Haskins (1960), B/r is resistant to penicillin and to sulphathiazole as well as to radiation. None of the R-types of S was resistant to penicillin and none of those tested (R_1 to R_4) was resistant to sulphonamides (unpublished). Moreover, while B/r, R_3 and R_4 do not, as do B and S, respond to the challenge of radiation by forming filaments, filament formation after irradiation is characteristic of R_1 and R_2 (Curry & Greenberg, unpublished).

Assuming all the R-types of strains S are each due to a single mutation, there may be as many as 17 mutations involved in the resistance or sensitivity of *Escherichia coli* to radiation. Witkin (1947) recognized four different groups of radiation-resistant mutants of *E. coli* B. One group, represented by B/r, was resistant to penicillin and sulphathiazole; another was resistant to penicillin but not sulphathiazole; another was resistant to sulphathiazole but not to penicillin; and a fourth group was resistant to neither of these agents. Two of these mutants, the two with concomitant resistance to penicillin, are obviously different from any of the R-types of S. The other two radioresistant mutants of B cannot yet be clearly distinguished from all the R-types of S. Also not yet fully distinguishable from the R-types of S are the radiation sensitive mutants of B, described by Hill & Simson (1961) although B_s and B_{s-2} appear to be more sensitive than S. With respect to radiation sensitivity together with collateral resistance to chemical agents, *E. coli* B exists in at least seven forms, *E. coli* S in eight. All the forms of B might be counterparts of those in S, differing only in one mutation from B to S, or vice versa. If this were the case, there would be nine mutations controlling radiation sensitivity and collateral resistance to other agents in *E. coli*. If, on the other hand, none of the mutants of B were counterparts of any of those in S, and B and S were different, there might be as many as 16 mutational events controlling radioresistance to *E. coli*. Furthermore, B/r would appear to be about as resistant to radiation as the R-types of S, but *E. coli* H, is, according to our experience, about twice as resistant to ultraviolet radiation as the R-types of S. Assuming, then, that a single mutation controls the difference between S and H, with respect to radiation sensitivity, there may be as many as 17 mutational events controlling radiation sensitivity in *E. coli*. By integrating the published data with our own experience we would estimate that the difference between the most radiation sensitive and resistant forms of *E. coli* (B_s of Hill & Simson, 1961, and H) in resistance to ultraviolet radiation to be about 200-fold.

The isolation of five consecutive steps in resistance to Mitomycin C appears to reflect the interaction between radio- and chemoresistant mutations. S/Mc 2a may be considered to consist of an R_3 plus a chemoresistant mutation similar to or identical with S/Mc 1c. The third-step mutant, S/Mc 3a, probably represents a shift from R_3 to R_4 , superimposed on its chemoresistant mutation acquired in the previous step. Previously it was clear that no R-group was a prerequisite for any other R-group; that is, all the R-groups could be selected in one step from the parent sensitive strain. Furthermore, in earlier experiences with nitrosoguanidines, nitro-min or nitrogen mustard no R-group was selected from any other R-group. It was as though all R-types were mutually exclusive. Now it is evident from the change in resistance patterns from S/Mc 2a to S/Mc 3a that mutations can occur from one R group to another. Such a shift would appear to require a single genetic change. S/Mc 4a and S/Mc 5a, in turn, may represent either the imposition of additional

chemoresistant steps on a basic R_4 radioresistance or a change from R_4 to R_7 . It should be noted that as many as three of the five steps in resistance to Mitomycin C may represent chemoresistant mutations, but only two such mutations were identified as first-step mutants. This might mean that certain chemoresistant mutations can occur only after another one has occurred; or that among the first-step chemoresistant mutants there are two phenotypes for three genotypes; or that one chemoresistant mutation has not yet been discovered; or, as suggested above, one of the steps is a change from R_4 to R_7 .

It is evident from this discussion that there are many different radioresistant mutants of *Escherichia coli*, each with a characteristic cross-resistance pattern to ultraviolet radiation, a variety of radiomimetic and some non-radiomimetic agents. Genetic analyses of these mutants are planned, for without such an analysis it is difficult to determine whether the mutants represent different alleles at one locus or mutations at different loci. Information of this nature will be a useful guide for postulating models to account for resistance to the lethal effects of radiation and radiomimetic chemicals.

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The Role of Polygalacturonase in Root-Hair Invasion by Nodule Bacteria

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SUMMARY

The production of polygalacturonase in associations of nodule bacteria and seedlings of leguminous plants was investigated. Plants and bacteria were combined in the following ways: (a) different plant species were combined with infective strains (isolated from the same cross inoculation group) as well as non-infective ones (from foreign groups); (b) host plant species with different susceptibilities were combined with the same bacterial strain; (c) bacterial strains with different infectivities, as measured by the number of infection sites, were combined with the same host species; (d) clover strains which had lost their infectivity, as well as transformed and again infective subcultures of these strains, were tested on clover plants. The results indicate that infection of the seedlings was strongly correlated with the production of polygalacturonase. The conclusion is drawn that polygalacturonase plays an important part in the infection process. This function is thought to be a weakening of the cell wall of the root hair which would facilitate the bacterial invasion. The possible role also of indolyl-3-acetic acid in the infection process is discussed.

INTRODUCTION

In a study of the symbiotic relationships between leguminous plants and nodule bacteria one of the first questions which arises is how the bacteria enter the plant. This problem has been comparatively little studied, and no clear picture has so far been obtained of this important step in the development of the symbiosis. In previous work (Fåhræus & Ljunggren, 1959) some evidence was found that pectic enzymes take part in the infection process. Significant amounts of polygalacturonase were found in associations of seedlings and their specific nodule bacteria, but not in those combinations where no infection occurred. An addition of nitrate, which stops the infection, also inhibited polygalacturonase production. Polygalacturonase was produced by the plant, not by the bacteria, which appears from the fact that an addition of cell-free preparations from the bacteria had the same effect as living bacteria in inducing formation of polygalacturonase (Ljunggren & Fåhræus, 1959). Since in the above-mentioned work only rather few combinations of plants and bacteria had been examined, the investigation was extended by testing a larger number of plant species and bacteria. Bacterial strains with different degrees of infectivity and plant species with different susceptibilities were also studied. The present paper presents the results obtained, which seem to confirm the importance of the polygalacturonase in the infection process. On the basis of these findings a possible mechanism for rhizobial infection is discussed.

METHODS

Seed material. Commercially available seed of the following plants was used: *Trifolium repens* L. (varieties Morsö and Beta), *T. hybridum* L. (variety Svea), *Medicago sativa* L. (varieties Tuna and Alfa), *Pisum sativum* L. (variety Torsdags II). These were ordered from Svalöv, except *M. sativa*, variety Alfa, which was obtained from Weibullsholm, Sweden. In addition, several wild species were used for the experiments. The seed had been collected by Dr P. S. Nutman, who kindly put it at our disposal. These species were: *Trifolium ambiguum* M. Bieb., *T. glomeratum* L., *T. parviflorum* Ehrh., *T. patens* Schreb., *Melilotus alba* Desv., *Vicia hirsuta* (L.) S. F. Gray, *Lotus angustissima* L., *L. hispida* Desf., *Anthyllis vulneraria* L.

Bacterial strains. *Rhizobium trifolii*: strains 226, ClF, A 121111, SU 297, all infective and effective nitrogen fixers; strain Coryn, infective but forming ineffective nodules; strains Bart A and A 11, non-infective variants of originally infective strains. It has been possible to transfer infectivity to these strains by adding capsular material produced by the infective strain 226 (Ljunggren, 1961). The transformed strains are called Bart A2 and A 11.2, respectively. Other strains: *R. meliloti* AH₂, *R. leguminosarum* 311 and V1, *Rhizobium* sp. (Lotus) L1, *R. japonicum* 507.

The strains 226 and 311 were obtained by the courtesy of Dr G. Bjälfve (Leguminous Plant Laboratory, Uppsala); the others were generously supplied by Dr P. S. Nutman.

Substrates and chemicals. For cultivation of *Rhizobium* the medium A₅ given by Dorn (1956) was used throughout. For sterility control a standard Difco nutrient agar was used. The pectin used in the determinations of polygalacturonase was low-methoxyl citrus pectin Matheson, Coleman and Bell no. 7366 (Norwood, Cincinnati, Ohio, U.S.A.).

Experimental procedure

The experimental procedure was essentially the same as reported in a previous paper (Fähræus & Ljunggren, 1959). Some minor changes in the technique and details which we think are of special importance for the successful performance of the experiments are given in the following paragraphs.

For seed disinfection a HgCl₂+formaldehyde mixture (Fähræus, 1957) was ordinarily used. Extreme care was exercised to remove traces of mercury and possibly harmful seed exudates (Dadd & Jacobs, 1958; Jacobs & Dadd, 1959). To this end, the seed was allowed to swell overnight in the last wash water and was then rinsed again several times. For hard-coated seeds the procedure with sulphuric acid (Nutman, 1959) was more satisfactory and it was used for *Trifolium pratense*, *T. ambiguum*, *T. subterraneum*, *Lotus hispida*, *Anthyllis vulneraria*, *Melilotus alba* and *Vicia hirsuta*.

Certain batches of seed were very difficult to disinfect by any of these methods. Better results were sometimes obtained when the seed after disinfection was allowed to germinate in 0.01 % (w/v) hydroxylamine (personal recommendation by Dr G. Bjälfve). Nevertheless, in many cases we obtained vigorous growth of a Gram-negative, non-sporulating rod which gave yellow colonies on nutrient agar, it was probably a *Flavobacterium*.

Following disinfection the seed was transferred to sterile 10 cm. diam. Petri dishes, and the water volume adjusted to 2-3 ml. in each dish. Usually on the

second day the seedlings were inoculated with *Rhizobium* suspensions made up in distilled water. The suspensions were prepared from agar slopes with 3-day bacterial growth; 0.5 ml. containing about 500 million organisms/ml. was added to each dish, the total volume being adjusted to about 5 ml. with sterile distilled water. The use of distilled water seems to be an important point in this type of work, because even traces of Ca disturb the viscometric analysis. For that reason it is also advisable to rinse all glassware carefully in distilled water.

The dishes after inoculation were kept in an incubator at $20^{\circ} \pm 1^{\circ}$, and were illuminated with fluorescent light.

The experiments were terminated 3–4 days after inoculation. The solution was separated from the roots and solid particles by filtration through a layer of cotton wool.

For the determination of polygalacturonase the viscometric technique described previously (Fåhræus & Ljunggren, 1959) was used throughout. The pectin was dissolved in 0.1 M-acetate buffer (pH 5.0) containing 0.5 % (w/v) NaCl. The solution was filtered on a Buchner funnel through double papers to free it completely from suspended particles. Together with the enzyme solutions to be tested the pectin solution was kept in a constant temperature bath at 30° for temperature equilibration. At zero time the solutions were mixed in Ostwald viscometers (3 ml. test solution + 8 ml. substrate in buffer) and the flow time recorded immediately. Measurements were again made after 1 hr. and 24 hr. The viscometers used had a flow time for water (t_w) between 31 and 35 sec. To get the t_w -value for a 'standard viscometer' with a flow time of 40 sec. we multiplied the values obtained with the different viscometers by the factor $k = 40/t$, where t represents the flow time of water in the individual viscometers.

The decrease in viscosity given in percentage of initial values was calculated from the expression

$$P = \frac{t_0 - t_t}{t_0 - t_w} \times 100,$$

where P = percentage decrease in viscosity; t_0 = initial flow time; t_t = flow time after t min.; t_w = flow time of water.

Sterility control. Since pectin-decomposing micro-organisms are common and the presence of contaminants especially of this kind in our experiments would largely invalidate the results, it was of the utmost importance to keep infection hazards as low as possible. The following precautions were therefore strictly followed.

(a) When the seed had been transferred to Petri dishes for germination, about 50 seeds from the disinfection flask were distributed over two plates of nutrient agar. When microbial growth occurred on these plates, the whole experiment was discarded.

(b) Immediately before inoculation, nutrient agar plates were streaked with liquid from each dish. All dishes which showed microbial growth were discarded.

(c) Before taking samples for analysis, the liquid and a number of roots were examined microscopically. Uninoculated dishes which showed microbial growth and inoculated dishes with growth morphologically distinct from *Rhizobium* were discarded. Fungal mycelium, when present, usually grew along the roots and was easily observed microscopically.

RESULTS

Cross-infection group specificity

In a series of experiments leguminous seedlings were combined with different species and strains of *Rhizobium*. As some seeds were available in small quantities only, experiments could not always be performed to determine the most suitable period for incubation. As a rule, however, satisfactory results were obtained by inoculating the dishes when the seedlings were 8–10 mm. long and incubating for another 3–4 days. The experiments are summarized in Table 1, from which it is evident that polygalacturonase was always present, when the seedlings were inoculated with the appropriate bacteria and that there was no, or very little, activity in other combinations. In this connexion it is interesting to notice Nutman's (1959) report that the *Lotus* and *Anthyllis* species showed deformed root hairs and produced nodules but that no infection threads were observed. In our test, however, they did not significantly differ from the other species tested.

Table 1. *Formation of polygalacturonase (PG) in associations of leguminous plants and nodule bacteria*

PG activity expressed as % decrease in viscosity of a 1 % (w/v) solution of low-methoxyl pectin in 24 hr.

Rhizobium strains	Plant									
	<i>Trifolium repens</i>	<i>Trifolium ambiguum</i>	<i>Trifolium hybridum</i>	<i>Medicago sativa</i>	<i>Medicago alba</i>	<i>Pisum sativum</i>	<i>Vicia hirsuta</i>	<i>Lotus hispida</i>	<i>Lotus angustissima</i>	<i>Anthyllis vulneraria</i>
	PG activity									
Clover { CIF	12.0	—	—	—	2.0	—	—	—	—	—
226	16.1	—	11.0	—0.8	—	0.0	3.5	3.1	—	1.9
A121111	25.4	10.6	—	—	5.6	—	—	—	0.8	—
Lucerne AH ₂	1.0	0.5	—	22.2	20.6	—	—	—	3.7	—
Lotus L1	2.8	2.3	—	1.0	—	—	3.5	14.3	46.6	12.5
Vetch V1	2.0	—	—	2.4	—	—	16.6	0.9	—	0.5
Pea 311	1.2	—	—	—	—	16.4	—	—	—	—
Soybean 507	1.1	—	—	—	—	—	—	—	—	—

Host/bacteria effects within the clover group

Host differences. Nutman in a series of papers (reviewed in Nutman, 1956) studied the genetic factors affecting host/bacterial compatibility. An examination of infection density was also performed by Nutman (1959), but in this case without genetical analysis. An examination of his data, however, shows that there was a difference in infection density between different host species inoculated with the same bacterial strain. For instance, the mean number of infected hairs/plant after 11 days was 91.5 in *Trifolium parviflorum* and 7.0 in *T. patens*, when inoculated with *Rhizobium* strain CIF. The amounts of polygalacturonase produced by the above-mentioned hosts inoculated with one bacterial strain is shown in Table 2. The results indicate that there was a higher polygalacturonase activity in the more susceptible plant species.

Bacterial strain differences. In his work on root hair infection by nodule bacteria Nutman (1959) found great differences in infection density in the same host plant when inoculated with various strains of *Rhizobium trifolii*, and stated that these differences were independent of the host. Table 3 shows the results of two experiments with *Trifolium repens* and *T. glomeratum* inoculated with different strains of clover bacteria. The number of infection threads on the same level on both sides of the root were counted by using the slide technique earlier described (Fåhræus, 1957) and were compared with polygalacturonase activities found in simultaneous experiments. From Table 3 it is evident that there was a certain correlation between the number of infection threads and the polygalacturonase activity found.

Table 2. *Formation of polygalacturonase (PG) by two species of Trifolium inoculated with the same bacterial strain.*

PG activity as % decrease in viscosity of a 1 % (w/v) solution of low-methoxyl pectin in 24 hr.

Host plant	Rhizobium strain	PG activity
<i>Trifolium parviflorum</i>	—	1.4
	CIF	27.6
<i>T. patens</i>	—	0.9
	CIF	14.9

Table 3. *The relation between polygalacturonase (PG) activity and number of root-hair infections in two species of Trifolium in association with different strains of Rhizobium trifolii*

PG activity as % decrease in viscosity of a 1 % (w/v) solution of low-methoxyl pectin in 24 hr.

Host plant	Rhizobium strain	PG activity		Mean number of infected root hairs
<i>Trifolium repens</i>	—	—0.7	1.4	0
	CIF	11.2	12.2	3.5
	Coryn	17.6	22.7	18.5
	226	17.0	23.0	21.5
<i>T. glomeratum</i>	—	2.0	—	—
	CIF	16.0	—	48.6*
	S.U. 297	11.6	—	9.8*

* Figures from Nutman (1959).

Experiments with non-infective clover strains. The origin of the *Rhizobium* strains Bart A, A11 and A121111 was recorded by Nutman (1946). Of these three strains Bart A and A11 have lost their ability to invade the root hairs of their original host plants and hence they are no longer able to induce nodule production. Table 4 shows that the non-infective bacteria were unable to induce polygalacturonase production. In this respect there was no difference between them and quite unrelated bacteria. We have, however, been able to transfer infectiveness to these bacteria from the infective strain 226 by adding capsular material from the latter to seedlings planted on an agar slope and inoculated with *Rhizobium* strains Bart A and A11. These seedlings produced nodules, and the bacteria isolated from these nodules (Bart A2, A11.2) are infective (Ljunggren, 1961). As shown in Table 4, the transformed

strains are also active polygalacturonase producers. These experiments have in fact given the only unequivocal proof, except serological tests, that Bart A and A11 really are *Rhizobium* strains.

Table 4. *Formation of polygalacturonase (PG) by Trifolium repens in association with infective and non-infective strains of Rhizobium trifolii*

PG activity as % decrease in viscosity of 1 % (w/v) solution of low-methoxyl pectin in 24 hr.

Rhizobium strain	PG activity	
	Expt. 1	Expt. 2
—	—1.0	0.5
Bart A	1.5	2.1
A11	2.5	1.1
A121111	15.0	15.2
226	16.2	15.4
Bart A2	—	10.8
A11.2	—	11.3

DISCUSSION

The correlation found between the infection of leguminous plants by nodule bacteria and the increased production of polygalacturonase in the bacteria+plant associations can be interpreted in two ways: (i) polygalacturonase formation may be the primary effect of bacterial action, (ii) the infection might come first and lead to an increased production of polygalacturonase. We have drawn the conclusion that the formation of polygalacturonase is the primary effect resulting in a partial depolymerization of the cell-wall pectin which, in its turn, facilitates the bacterial invasion. For this succession of events speaks the fact that infection rarely can be observed until after 3 days, whereas an increased production of polygalacturonase may occur as soon as 1 to 2 days following inoculation.

The infection mechanism may be visualized in the following manner. The bacteria secrete water-soluble substances which are highly specific (Humphrey & Vincent, 1959; Ljunggren & Fåhræus, 1959). Our active preparations are mainly of polysaccharide nature, but transformation experiments carried out by one of us (Ljunggren, 1961) suggest that they might also contain deoxyribonucleic acid. The active principle will pass through the cell wall, reach the protoplasm, and react with some specific cell component. The nucleus, which at this stage is situated in the root hair near the tip, may possibly be involved in the interaction with the bacterial compound. Previous observations (Fåhræus, 1957; Nutman, 1959) suggest that the nucleus takes an active part in the infection process, but the mechanism is still obscure.

The reaction between the bacterial substance and the specific cell compound results in the formation of an 'organizer' which governs the production of polygalacturonase. This enzyme is always present in the growing root hair in small amounts. The apical hair wall consists mainly of pectic substances, and the function of polygalacturonase may be a continuous softening of this wall, which leads to cell elongation (Ekdahl, 1953). However, when appropriate nodule bacteria are present, they induce, in some hairs at least, a much stronger production of polygalacturonase. This might result in a more pronounced depolymerization of the pectic layer, which

would allow the bacteria to penetrate the cell wall. But it appears also possible that the increased polygalacturonase activity can result in an intensified but very localized growth process giving an infection thread by invagination, in accordance with Nutman's hypothesis (1956). It seems to us that an important role in the infection process can be ascribed to polygalacturonase regardless of whether Nutman's hypothesis be accepted or not.

The mechanism outlined in this way could explain the high specificity which is characteristic for the cross-inoculation groups of leguminous plants, since the

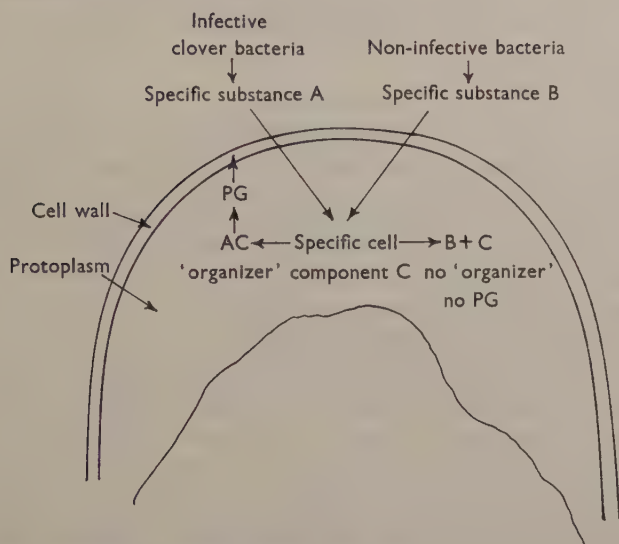


Fig. 1. Induced formation of polygalacturonase (PG) in root hair tip of clover plant.

specificity of polygalacturonase formation, as shown by our experiments, is quite as high. This is further illustrated by the tentative scheme given in Fig. 1. A prerequisite for infection as well as for polygalacturonase production is a certain nitrogen deficiency (Fåhræus & Ljunggren, 1959). The reason why polygalacturonase formation is stopped at higher nitrate concentrations might perhaps be that nitrogen stimulates synthetic mechanisms in the plant which decrease the formation of depolymerizing enzymes. Even at suboptimal nitrogen concentrations, the increased polygalacturonase activity is probably not a sufficient condition for infection to take place. The well known fact that only a very restricted number among thousands of root hairs are invaded indicates that the infection is a rather complex process. Possibly the cell wall must in some way be 'sensitized' before the polygalacturonase becomes active. It lies near at hand in this connexion to think of indolyl-3-acetic acid (IAA). IAA increases the plasticity of cell walls (Galston & Purves, 1960), and is produced by nodule bacteria (Link, 1937; Kefford, Brockwell & Zwar, 1960). According to Thornton (1936) the deformation of root hairs, which is believed to be caused by bacterial IAA, is a necessary prelude to infection. However, our present knowledge of the action of IAA on cell walls—which might involve an immobilization of pectin methylesterase (Glasziou, 1957; Fåhræus & Ljunggren, 1959)—are too vague to permit detailed conclusions about the role of

IAA in infection. It is certainly not possible to explain the whole infection process only as an IAA effect (Kefford, Brockwell & Zwar, 1960). Such an explanation does not account for the marked specificity of rhizobial infection, since IAA is produced by almost all types of nodule bacteria, and also by *Agrobacterium radiobacter* which is unable to infect any leguminous plant (Georgi & Beguin, 1939).

A large part of this investigation was made during a visit by one of us (H.L.) at the Rothamsted Experimental Station, Harpenden, Hertfordshire. Thanks are due to the Head of the Soil Microbiology Department, Dr P. S. Nutman, for his great hospitality and interest in the work. Financial support was obtained from M. Bergvall's foundation, Stockholm.

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The Isolation of [^{35}S]Homocystine from *Neurospora*

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SUMMARY

Evidence is presented inferring accumulation of [^{35}S]homocystine in methionineless mutants of *Neurospora crassa*. Certain mutants formed five times as much homocystine as did the wild type; [^{35}S]homocystine was identified as homocystine and homocysteic acid.

INTRODUCTION

Extensive studies have shown the physiological activity of homocystine in replacing methionine (Butz & Du Vigneaud, 1932; Horowitz, 1947, 1950; Shapiro, 1955; Schlenk & DePalma, 1955; Durell, Anderson & Cantoni, 1957). Thus the role of homocystine as an intermediate in methionine biosynthesis has become accepted although it has never been isolated from biological material. Because homocystine usually occurs only as a transitory intermediate in methionine biosynthesis it was felt that methionineless mutants of *Neurospora crassa* might accumulate homocystine and would be particularly useful in attempts to show directly the occurrence of this compound. The experiments described below show that homocystine occurs in *N. crassa* and in greater concentration in these mutants than in wild type, as reported in a preliminary note (Leinweber & Liverman, 1959). After this work was completed the authors learned of a communication on the isolation of homocystine from the human adrenal gland (Biserte, 1957); quantitative data, however, were not reported.

METHODS

Wild type strain 5297a and mutants 38706Ra and 80702Ra of *Neurospora crassa* were obtained from Dr M. Fling, Division of Biology, California Institute of Technology, Pasadena, California, U.S.A. Mutants T-27 and T-112 originated in the Genetics Laboratory of the University of Texas and were obtained from Dr Robert A. McRorie, University of Georgia, Athens, Georgia, U.S.A. Mutants 38706Ra, T-27 and T-112 do not grow on any precursor of methionine biosynthesis (Horowitz, 1947, 1950) but require methionine for growth. They are believed to lack an enzyme necessary for the biosynthesis of methionine from homocystine. With regard to mutants T-27 and 38706Ra, at least two differences are known. Growth of mutant T-27 on methionine is enhanced by augmenting sulphate in the medium; in mutant 38706Ra, sulphate has no effect on growth in the presence of methionine. S-methyl-

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L-cysteine can serve as sole sulphur source for mutant T-27 but not for 38706Ra (Ragland & Liverman, private communication). Mutant 80702Ra does not grow on sulphate or sulphite and was proposed to have a genetic block between sulphite and thiosulphate (Horowitz, 1950).

The various strains of *Neurospora crassa* were grown on 20 ml. of sulphur-free minimal medium contained in 125 ml. Erlenmeyer flasks incubated at 25°. The medium was prepared by replacing the sulphates of the Difco Choline Assay Medium (Difco Manual, 1953) by the chloride salts in equimolar amounts with respect to the cation. Methionine was supplemented to all strains in a concentration of $5 \times 10^{-4}M$ which was found optimal for growth and was added aseptically after filter-sterilization. Radiosulphate, purchased from the Oak Ridge National Laboratories, was added either carrier-free or with non-radioactive potassium sulphate. The final concentration of radiosulphate in the medium was 8–15 $\mu c./ml.$, the specific radioactivity 150–174 $\mu c./\mu mole$ unless otherwise stated.

At the end of the growth period, the mycelium was harvested by filtration, freed from culture medium by suction, and repeatedly washed with distilled water. The filtrate was discarded unless otherwise stated. The mycelium was ground in a mortar with sand and 10 ml. of cold 10% (w/v) trichloroacetic acid (TCA), and the homogenate centrifuged. The pellet was twice resuspended and washed with 10 ml. of 5% (w/v) TCA and again centrifuged. The supernatant fluids were pooled and accurately weighed amounts of homocysteine (e.g. 1.5 m-mole; purchased from Nutritional Biochemicals Corporation, 21010 Miles Ave., Cleveland, Ohio, U.S.A.) + homocystine (0.75 m-mole; purchased from California Corporation for Biochemical Research, 3625 Medford St., Los Angeles 63, California, U.S.A.) were added in order to isolate [^{35}S]homocysteine formed in the mycelium. The homocysteine in the TCA extract was then oxidized to the disulphide by the following procedure which was developed in this laboratory. The extract was adjusted to pH 8–9 with sodium hydroxide solution. One drop 0.3M solution of ferric chloride was added per m-mole of homocysteine-carrier, and oxygen bubbled through the solution until oxidation was complete, as indicated by fading of the violet colour of the iron-sulphydryl complex. The solution was then adjusted to pH 6 to precipitate homocystine. Any contaminating protein was removed by dissolving the precipitated homocystine in 7 ml. of 2N-hydrochloric acid and dialyzing against 50 ml. of 0.5N-hydrochloric acid. The cellophan sack and its remaining contents were discarded. The homocystine in the diffusate was precipitated by adjusting the solution to pH 6. After dissolving the homocystine in a minimum amount of dilute hydrochloric acid it was further purified by subjecting to paper electrophoresis, using a Spenco Continuous Flow Electrophoresis Apparatus, Model CP, at 5°–10°. The homocystine separated as a narrow band when the electrophoresis was carried out at 900 V. in 0.25M-acetic acid. The homocystine samples thus obtained were recrystallized until the specific radioactivity and the decomposition temperature became constant. Chromatographic assays of the re-isolated homocystine were performed, using four different solvent systems, in one and in two dimensions. The solvent systems used were: (1) methyl ethylketone + *n*-butanol + distilled water + dicyclohexylamine (10 + 10 + 5 + 2); (2) 3-butanol + 90% (w/v) formic acid + distilled water (70 + 15 + 15); (3) phenol + de-ionized water + 0.88 N-ammonia (160 + 40 + 1); (4) *n*-butanol + distilled water + pyridine (1 + 1 + 1).

RESULTS

In a preliminary experiment it was found that *Neurospora crassa* methionineless mutants 38706Ra and T-27 formed [^{35}S]homocystine. The concentrations of [^{35}S]homocystine were rather low. It was, therefore, attempted to enhance the formation of [^{35}S]homocystine by adding non-radioactive sulphate to the medium. In this experiment homocystine was re-isolated from the combined TCA extract and the filtered culture fluid after 9 days of incubation. At a concentration of $8.6 \times 10^{-5}\text{M}$ -carrier-sulphate the [^{35}S]homocystine concentration, expressed as amount [^{35}S]homocystine/g. dry mycelium, approximately doubled as compared to that obtained in the absence of potassium sulphate, as shown in Fig. 1. Further increasing the concentration of carrier-sulphate does not give rise to higher concentrations of [^{35}S]homocystine; since, however, a 3.3-fold increase in carrier-

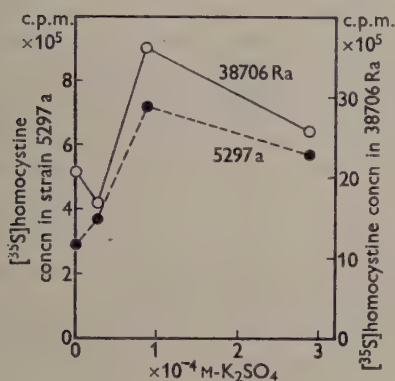


Fig. 1

Fig. 1. [^{35}S]homocystine formation in *Neurospora crassa* as function of sulphate concentration in the culture medium. Left scale refers to wild type strain 5297a, right scale to mutant 38706Ra. The mycelium was incubated for 9 days. Specific radioactivity of sulphate at $8.6 \times 10^{-5}\text{M} = 98 \mu\text{c.}/\mu\text{M}$.

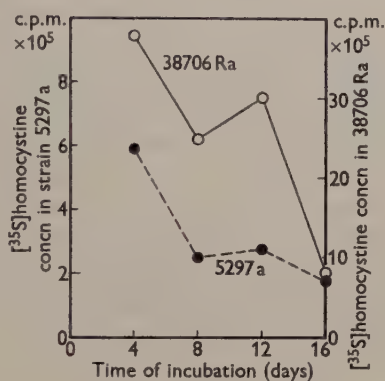


Fig. 2

Fig. 2. [^{35}S]homocystine formation in *Neurospora crassa* as function of time of incubation.

sulphate concentration resulted in a decrease in [^{35}S]homocystine of only *c.* 25 %, the optimal sulphate concentration for homocystine formation is apparently higher than $8.6 \times 10^{-5}\text{M}$.

The data suggest that some optimal concentration of sulphate is required for homocystine formation. As mentioned under Methods and as shown below, sulphate alone does not promote any growth in mutant 38706Ra unless incubated for longer than 2 weeks. Growth, under the conditions employed, therefore, is a function of methionine concentration. Mutant 38706Ra accumulated five times as much [^{35}S]homocystine as did wild-type strain 5297a.

It was expected that increases in incubation time of the mycelia would enhance [^{35}S]homocystine accumulation. Wild-type and mutant 38706Ra were, therefore, incubated for 4, 8, 12 and 16 days in the presence of $5 \times 10^{-4}\text{M}$ -methionine, $8.6 \times 10^{-5}\text{M}$ -non-radioactive sulphate and $15 \mu\text{c.}$ of [^{35}S]sulphate/ml. medium. As shown in Fig. 2, the maximum accumulation of [^{35}S]homocystine, as measured in the TCA

extract, was noted after four days of growth. Prolonging the incubation period resulted in lower [^{35}S]homocystine concentrations. It is of interest to note that between the 4th and 8th day of incubation the [^{35}S]homocystine concentration in mutant 38706Ra decreased while the mycelial dry weight increased by 58 %, indicating that the decrease in [^{35}S]homocystine concentration is not related to depletion of methionine in the culture fluid. Considering the difference in the ordinate scale of a factor of 4 for the plot of mutant and wild type, it can be seen in both figures that the mutant formed 5–11 times as much [^{35}S]homocystine as did the wild type. Measurements of [^{35}S]methionine formation were not made; the results suggest, though, that the block preventing methionine biosynthesis from homocysteine is shown to be incomplete if the incubation period is extended beyond 4 days. In an experiment to test this postulate mutant 38706Ra was grown in parallel on $2 \times 10^{-4}\text{M}$ -methionine or $2 \times 10^{-4}\text{M}$ -potassium sulphate for 4, 12, 17 and 22 days. After 4 days there was no growth on sulphate but after 12, 17 and 22 days the growth was 8, 50 and 86 % respectively of that on methionine. This experiment strongly supports the above postulate. In a mutant of *Neurospora crassa* which is blocked between cystathionine and homocysteine and which grows only on homocysteine or methionine it was demonstrated that methionine biosynthesis from [^{35}S]sulphate takes place after prolonged incubation (Strauss & Minagawa, 1959); [^{35}S]cystathionine first accumulated, and later disappeared as [^{35}S]methionine was formed.

Homocystine formation in mycelia of mutant T-112 and 80702Ra and wild-type strain 5297a was compared. It was found that mutant T-112 formed 4.8 times as much [^{35}S]homocystine as did wild type, whereas mutant 80702Ra formed only 0.58 times as much. Such a result might be expected since mutant 80702Ra is blocked before homocysteine in the biosynthetic pathway. The observation that homocystine re-isolated from this mutant was slightly radioactive might indicate that the metabolic block is not complete.

A chromatographic study confirmed that the radioactivity measured in mutants 38706Ra, T-27 and T-112 was due to [^{35}S]homocystine. The ninhydrin-positive material always coincided in size, shape and location with the black spot on the radioautograms. The nitroprusside test for disulphide compounds (Toennies & Kolb, 1951) also supported the identity of the radioactive material as homocystine. Furthermore, when homocystine was oxidized to homocysteic acid with 30 % (w/v) H_2O_2 before developing the chromatograms, only one black spot appeared on the film which coincided with the ninhydrin-positive material on the paper. Because the specific radioactivity of homocystine from wild type was too low we were unable to obtain radioautograms in this case. It seems, however, reasonable to attribute the radioactivity measured in homocystine from wild type to the formation of some [^{35}S]homocystine.

The chromatographic techniques used permit differentiation of homocystine from other sulphur amino acids, e.g. methionine, cysteine, cystine, cystathionine, lanthionine, djenkolic acid. Although homocysteine was oxidized during the isolation procedures it is probable that the [^{35}S]homocystine finally identified was due to formation of [^{35}S]homocystine in the mycelium.

Since varying the growth period or the sulphate concentration never resulted in [^{35}S]homocystine yields larger than 0.3 % in terms of ^{35}S initially present in the

medium, no attempts were made to isolate homocystine without employing non-radioactive homocystine-carrier.

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The Relationship of the Enterobacterium A12 (Sachs) to *Shigella boydii* 14

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SUMMARY

The relationship of the enterobacterium A12 (Sachs) to *Shigella boydii* 14 is shown by comparison of their biochemical and serological reactions. The aerogenic, mannitol-negative A12 is serologically identical with the typical *Shigella* serotype, *S. boydii* 14. As mannitol-negative strains of *S. boydii* 14 exist and as the aerogenic A12 organism is otherwise biochemically typical of the *Shigella* group, it is proposed that A12 be regarded as a biochemical variety of *S. boydii* 14 analogous to the accepted aerogenic biochemical varieties of *S. flexneri* 6.

INTRODUCTION

It is well known that there is a wide sharing of antigens throughout the whole family of the Enterobacteriaceae so that the determination of the antigenic structure of a particular enterobacterium is only one part of the jigsaw of identification and subsequent classification. The identification of these organisms must, at least at present, be based on both biochemical and serological criteria. Even so bacteriologists constantly encounter organisms which cannot easily be placed in one or other of the groups of the Enterobacteriaceae, whose main subdivisions, with the increasing use of the more detailed biochemical tests, are now reasonably well defined.

The aerogenic enterobacterium A12 of Sachs (1943) is an example of an intestinal organism whose exact taxonomic position within the Enterobacteriaceae is still not finally determined. Sachs considered this organism a possible member of the non-mannitol-fermenting subgroup of *Shigella*, whereas Ewing & Hucks (1950) considered it an aberrant coliform of the *Escherichia coli* O group 32. Wheeler & Stuart (1946) also discussed A12 and though, in the light of available knowledge at that time, they considered that the organism should not be included in the *Shigella* group, they did suggest that if a serological relationship to other strains of shigella was shown in time and if anaerogenic variants could be produced from it then there would be good reason to include it in the *Shigella* group. These authors drew attention to a similar situation occurring in the biochemical varieties of the Newcastle-Manchester-Boyd 88 dysentery bacilli. This opinion has now been fully justified with further knowledge and it is suggested that the position of A12 can be opportunely reviewed. It is proposed that this organism be regarded as a biochemical variety of the serotype *S. boydii* 14.

SOURCE OF ORGANISMS

There seem to be few records in the literature of the isolation of A12 from either single cases or outbreaks. In the first description of the organism Sachs examined 10 strains isolated in military laboratories on the North West Frontier of India between 1936 and 1941. The single A12 strain examined by Wheeler & Stuart was one of Sachs original strains and Ewing & Hucks strains are described as 'representative cultures', but these authors make no mention of freshly-isolated strains. Since the establishment of the Dysentery Reference Laboratory in 1945 21 strains of A12, freshly isolated in this country, have been identified. One strain was isolated in 1953 from a 14-year-old girl who had just arrived from the Sudan suffering from diarrhoea and from whom *Salmonella uganda* had also been isolated. Nineteen strains had been isolated during an outbreak of dysentery in a mental hospital in 1957; 16 of these strains were isolated from elderly female patients with acute diarrhoea, 1 from a 37-year-old patient who was herself probably infected in India and who undoubtedly introduced the infection to the hospital, and 2 were isolated from symptomless excretors (Dr H. I. Coombs, personal communication). One further strain was isolated from a 21-year-old male laboratory technician suffering from acute dysentery whose infection was acquired in the laboratory during the investigation of the 19 mental hospital strains.

METHODS

Strains. These 21 freshly isolated strains and the stock laboratory culture originally received from Sachs were available for detailed study in comparison with *Shigella boydii* 14. Six strains of *S. boydii* 14 were available and all have been received from Dr W. H. Ewing (Communicable Disease Centre, Atlanta, Georgia, U.S.A.) over the years 1952-57. Three strains of *Escherichia coli* O group 32 were also received from Dr Ewing.

Biochemical tests

Fermentation tests. Carbohydrates (0.5 %, w/v) in 1 % (w/v) peptone water with Andrade indicator, observed for 21 days.

Urease. Christensen (1946).

H₂S production. (1) Lead acetate papers over nutrient broth; (2) Triple Sugar Iron Agar (Test 10, *Report*, 1958*a*).

Phenylpyruvic acid test. (1) Henriksen (1950); (2) combined with malonate (Shaw & Clarke, 1955); (3) phenylalanine agar (Test 18, *Report*, 1958*a*).

Methyl red (MR) and Voges-Proskauer (VP) tests. Incubation for 5 days at 30°; VP test (O'Meara, 1931).

Citrate utilization. (1) Koser (1923); (2) Simmons (1926); (3) Christensen (1949) as modified by Edwards & Ewing (1955).

Malonate test. Combined with phenylalanine, Shaw & Clarke (1955).

Gluconate test. Shaw & Clarke (1955) modified by using 'Clinitest' Reagent Tablets (Ames Company, Nuffield House, London, W. 1) in place of Benedict qualitative solution.

Gelatine liquefaction. Nutrient gelatine stab culture incubated for 42 days at room temperature (about 22°).

Growth in KCN. Møller (1954) modified by using bijou bottles with the caps very tightly screwed.

Nitrate reduction. (1) 0.1 % (w/v) nitrate broth, tested after 5 days of incubation by Griess-Ilosvay method (Topley and Wilson's *Principles*, 1955); (2) plate test, Cook (1950).

Decarboxylases. Møller (1955).

Indole. 2 % (w/v) peptone water, (1) tested after 24 and 48 hr. with Kovacs reagent (1928); (2) oxalic acid papers (Holman & Gonzales, 1923).

Except as indicated, the temperature of incubation was 35°.

Serological tests

The antigen suspensions used were prepared in mercuric iodide solution (Bridges, 1951) from meat digest agar slopes and as necessary heated in a boiling water bath for 30 min. The several A12 and *Shigella boydii* 14 antisera used were prepared at the Dysentery Reference Laboratory. The two *Escherichia coli* O group 32 antisera used were kindly supplied by Dr W. H. Ewing and by Dr J. F. Winn (Communicable Disease Center, Atlanta, Georgia, U.S.A.). Tube agglutination tests were performed by a Dreyer technique and incubated in a 50° water bath for 4 hr., read, and incubated further overnight. All tests were done in duplicate with unheated and heated suspensions.

RESULTS

General characters. The A12 organisms are Gram-negative rods, non-sporing and non-acid-fast, indistinguishable in size and appearance from classical shigellas. Capsules have not been demonstrated. They are facultative aerobes and give characteristic shigella-like colonies on the usual 'enteric' media and, like shigellas, they do not grow on bismuth sulphite (Wilson & Blair) agar. Growth in broth and peptone water closely resembles that of accepted shigellas in showing a fairly clear supernatant fluid and a light powdery deposit, quite different from the denser uniform turbidity characteristic of the *Escherichia*, *Hafnia* or *Salmonella* groups.

Biochemical reactions

Table 1 summarizes the biochemical reactions of A12 and *Shigella boydii* 14. The volume of gas produced by the A12 strains from glucose occupied about $\frac{1}{4}$ – $\frac{1}{3}$ of the Durham tube; Sachs original strain produced less than the others but it is now an old laboratory culture. In general, only a tiny amount of gas, less than $\frac{1}{4}$ of the Durham tube, was produced from maltose. There was some minor strain variation in the period of incubation required for the fermentation of certain carbohydrates, but the average was fairly consistent. Xylose was generally fermented after incubation for 6 days, dextrin after 5 days, glycerol after 2 days and maltose after 5 days. Sorbitol was rapidly fermented, usually after 1–2 days. Dextrin was the most irregularly fermented carbohydrate. One of the A12 strains and two of the *S. boydii* 14 strains did not ferment it and most of the strains tested produced less than full acidity from it. On testing in Hugh & Leifson (1953) medium all strains showed fermentative metabolism.

Attempts were made by multiple colony selection to find anaerogenic colonies from the aerogenic strains. Though several colonies were found to produce only a tiny bubble of gas no colony failed to produce some gas from glucose.

Apart from the production or otherwise of gas, and the fermentation of mannitol

or otherwise, it is clear that the reactions of the A 12 organisms and *Shigella boydii* 14 are identical. None of the A 12 strains shows any biochemical reaction which would eliminate them from the *Shigella* group as at present defined (*Report*, 1958a).

Table 1. *Biochemical reactions*

	A 12		<i>S. boydii</i> 14	
	Sachs original strain	D.R.L. 21 strains	4 strains	2 strains
Glucose	AG	AG	A	A
Lactose	—	—	—	—
Mannitol	—	—	A	—
Sucrose	—	—	—	—
Dulcitol	—	—	—	—
Salicin	—	—	—	—
Xylose	(Ag)	(AG)	(A)	(A)
Adonitol	—	—	—	—
Arabinose	A	AG	A	A
Cellobiose	—	—	—	—
Dextrin	(a)	(a)/—	—/(a)	(A)
Glycerol	(A)	(A)	(A)	(A)
Inositol	—	—	—	—
Maltose	(Ag)	(Ag)	(A)	(A)
Raffinose	—	—	—	—
Rhamnose	—	—	—	—
Sorbitol	(AG)	(AG)	A	A
Trehalose	AG	AG	A	A
Urease production	—	—	—	—
H ₂ S production	—	—	—	—
Motility	—	—	—	—
Indole production	—	—	—	—
Phenylpyruvic acid test	—	—	—	—
MR reaction	+	+	+	+
VP reaction	—	—	—	—
Citrate utilization	—	—	—	—
Malonate	—	—	—	—
Gluconate	—	—	—	—
Gelatine liquefaction	—	—	—	—
Growth in KCN	—	—	—	—
Nitrate reduction	+	+	+	+
Oxidase	—	—	—	—
Catalase	+	+	+	+
Decarboxylases:				
Arginine	—	—	—	—
Lysine	—	—	—	—
Ornithine	—	—	—	—
Litmus milk	No change	No change	No change	No change

A = acid production within 24 hr.; (A) = acid after 48 hr.; (a) = weak acid; G = gas $\frac{1}{4}$ or more volume of Durham tube; g = gas less than $\frac{1}{4}$ the volume of Durham tube.

Serological reactions

In slide agglutination tests, all the strains of A 12, *Shigella boydii* 14 and *Escherichia coli* O group 32 examined agglutinated in each of the appropriate antisera for the three serotypes. None of the A 12 or *S. boydii* 14 strains showed any serological cross-relationship with other members of the *Shigella* group nor with Alka-

lescens-dispar O groups 1-4. The tube agglutination and absorption tests are summarized in Table 2.

The mirror absorption tests showed clearly that A12, *Shigella boydii* 14 and *Escherichia coli* O group 32 are serologically identical. This further confirms the work of Ewing & Hucks (1950) who showed that the somatic antigen of A12 was identical with that of *E. coli* O group 32 and of Ewing (1953) who stated that the then provisional *Shigella* serotype 2770-51 (now *S. boydii* 14) was identical serologically with *E. coli* O group 32.

Table 2. *Serological tests*

Titres are expressed as % of homologous titres for simplicity since more than one serum of each type and several strains were used, each serum giving a slightly different homologous titre.

Antiserum	Absorbing suspension	Antigen suspensions		
		A 12	<i>S. boydii</i> 14	<i>E. coli</i> O 32
A 12	—	100	100	100
	A 12	0	0	0
	<i>S. boydii</i> 14	0	0	0
	<i>E. coli</i> O 32	0	0	0
<i>S. boydii</i> 14	—	100	100	100
	A 12	0	0	0
	<i>S. boydii</i> 14	0	0	0
	<i>E. coli</i> O 32	0	0	0
<i>E. coli</i> O group 32	—	100	100	100
	A 12	0	0	0
	<i>S. boydii</i> 14	0	0	0
	<i>E. coli</i> O 32	0	0	0

DISCUSSION

In 1958 the provisional *Shigella* serotype 2770-51 was accepted as a typical member of the *Shigella boydii* subgroup and named *S. boydii* 14 (Report, 1958b). Ewing & Hucks (1952) in describing 2770-51 stated that 'it appears to us to be coincidental that serotype 2770-51 and type A12 (Sachs) both contain O antigens that are identical with those of *Escherichia coli* O group 32. Culture A12 is considered to be an aberrant intermediate paracolon bacterium because of its biochemical characteristics while serotype 2770-51 is believed to be a typical *Shigella* serotype with all the characteristics of *S. boydii* cultures.' They did not, however, consider the possibility that A12 might be a biochemical variety of *S. boydii* 14. The 2770-51 serotype was further discussed by Ewing, Reavis & Davis (1958) and they noted that six of their cultures (five from one familial outbreak) did not ferment mannitol.

Within the *Shigella* group only one member is at present accepted as having aerogenic varieties; this is the serotype *Shigella flexneri* 6. Scott (Whitehead & Scott, 1934) showed clearly that the aerogenic 'Newcastle' dysentery bacillus of Clayton & Warren (1929), the anaerogenic '88' dysentery bacillus of Boyd (1931) and the aerogenic 'Manchester' bacillus of Downie, Wade & Young (1933) were serologically identical. The biochemical reactions of this serotype are given in Table 3.

The Newcastle variety, which does not ferment mannitol, may or may not produce gas from glucose; this is like the aerogenic non-mannitol fermenting A12 and the

anaerogenic non-mannitol fermenting strains of *Shigella boydii* 14. To date, no strains of *S. boydii* 14 have been found which are both aerogenic and mannitol-fermenting, like the Manchester variety of *S. flexneri* 6. It is generally stated that the characteristic volume of gas produced from glucose by the aerogenic varieties of *S. flexneri* 6 is very small (*Topley and Wilson's Principles*, 1955) or sometimes described as a tiny bubble. This, however, appears from experience in the Dysentery Reference Laboratory to be no longer true. The aerogenic strains of *S. flexneri* 6 now produce a volume of gas quite as large as that produced by salmonellas. Even the original strains of Clayton & Warren, and Downie *et al.*, now produce a similar volume in the peptone water media in current use. There is some evidence that the use of modern peptones may influence the volume of gas produced by these organisms. The amount of gas produced by A12 is very similar to that of the aerogenic strains of *S. flexneri* 6. Table 4 shows the proposed biochemical varieties of *S. boydii* 14, analogous to those of *S. flexneri* 6.

Table 3. *Biochemical reactions of Shigella flexneri* 6

	Glucose	Mannitol	Dulcitol	Indole
Boyd's 88	A	A	A/—	—
Manchester variety	AG	AG	AG/—	—
Newcastle variety	AG/A	—	AG/A/—	—

Fermentation of dulcitol may be delayed.

Table 4. *Proposed biotypes of Shigella boydii* 14

	Glucose	Mannitol	Dulcitol	Indole
<i>S. boydii</i> 14 (classical)	A	A	—	—
<i>S. boydii</i> 14 (some strains)	A	—	—	—
A12	Ag	—	—	—

Pathogenicity. Though pathogenicity may be a poor criterion for taxonomy, the *Shigella* group as classified at present contains only organisms accepted as pathogenic and as causing bacillary dysentery. There seems to be no reason to exclude the A12 organism from the bacillary dysentery group. The mental hospital outbreak due to A12 shows that the organism is capable of causing an outbreak of dysentery and, though it might be said that elderly mental hospital patients may be in a special category of susceptibility to such infections, the infection of a young healthy laboratory technician during the handling of the cultures clearly indicates, in the absence of experimental animal infection, that the A12 organism is pathogenic and can produce a clinically typical attack of acute bacillary dysentery exactly similar to that of other shigella infections acquired in the laboratory (K. P. Carpenter, personal observation). Wheeler & Stuart's criteria in 1946 for acceptance of A12 as a member of the *Shigella* group have been fulfilled, and it appears expedient now that Sachs A12 be regarded as a typical aerogenic member of the group and as a biochemical variety of the serotype *Shigella boydii* 14, exactly analogous to the established biochemical varieties of *S. flexneri* 6.

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